Rôle du TGF-béta dans la carcinogenèse hépatique liée au virus de l'hépatite C

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Rôle du TGF-beta dans la carcinogenèse hépatique liée au virus de l'hépatite C

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À mes Parents

Résumé

L'infection chronique par le virus de l'hépatite C (VHC) conduit au développement de la fibrose et de la cirrhose qui risque d'évoluer vers le carcinome hépatocellulaire (CHC). La protéine de capside du VHC interagit avec de nombreuses protéines de l'hôte et en particulier avec Smad3, protéine majeure de la voie de signalisation du transforming growth factor beta (TGF-β). Mon travail de thèse consistait à étudier les conséquences biologiques de l'interaction entre la protéine de capside avec la voie de signalisation du TGF-β.

Le VHC présente une grande variabilité génétique et des travaux du laboratoire ont montré l'existence de séquences différentes de protéines de capside du virus entre les régions tumorales (cT) et cirrhotiques (cNT) d'un même sujet. Nous avons montré que ces différentes protéines de capside exprimées dans des hépatocytes orientent les réponses biologiques du TGF-β vers la promotion tumorale en diminuant l'apoptose et en augmentant la transition épithelio-mésenchymateuse (TEM) en particulier le variant cT. Cet effet est attribué à la capacité de la protéine de capside de diminuer l'activité transcriptionnelle de Smad3. De plus, les variants de la protéine de capside activent le TGF-β latent *via* l'augmentation de l'expression de la trombospondine.

L'un des marqueurs classiquement exprimé au cours d'une TEM est l'alpha-actine musculaire lisse (αSMA). Nous avons montré qu'une autre isoforme, la γSMA, était polymérisée dans les cellules hépatiques développant une TEM. L'expression de γSMA a été retrouvée sur des coupes de CHC et a pu être significativement corrélée à la fois avec des marqueurs de la TEM, des marqueurs progéniteurs et avec l'agressivité de la tumeur.

Ce travail apporte une meilleure compréhension du rôle de la protéine de capside dans la fibrose hépatique liée à l'infection virale. En effet, la protéine de capside du VHC agit à la fois de façon autocrine dans les hépatocytes en modulant les réponses du TGF-β vers la promotion tumorale et de façon paracrine, en affectant l'activation des cellules étoilées en myofibroblastes par le TGF- β activé.

Mots clés : Capside du VHC, TGF-beta, Transition Epithélio–Mésenchymateuse, Carcinome hépatocellulaire.

Abstract

Chronic HCV infection) may progress to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC). HCV core binds several cellular proteins and in particular Smad3, a major protein of transforming growth factor beta (TGF-β) signalling. The aim of this study was to determine the implication of HCV core protein in TGF-β responses.

High genetic variability is a characteristic of HCV and it was previously shown that HCV core protein isolated from tumour (cT) or adjacent non-tumour (cNT) livers displayed different sequences. Both were able to shift TGF-β responses from tumour suppressor to tumour promotor by decreasing hepatocyte apoptosis and increasing epithelial-mesenchymal transition (EMT). Core cT was more potent than core cNT to promote this effect that was mainly attributed to the capacity of HCV core to alleviate Smad3 activity. Moreover, HCV core protein activated the latent form of TGF-β through increased thrombospondin expression.

It is commonly accepted that αSMA (alpha smooth muscle actin) is a hallmark of EMT. In the current study another SMA isoform, γSMA was found to be polymerized during hepatocyte EMT. γSMA was expressed in HCC tissues and correlated with EMT, stem cell and aggressiveness markers.

In conclusion, this work contributed to a better understanding of the HCV core role in hepatitis fibrosis and HCC related to HCV. Indeed, HCV core might act both as an autocrine and paracrine way by modulating TGF-β responses within hepatocytes and by activating hepatic stellate cells in stromal environment through its capacity to activate TGF-β.

Keywords: HCV core, TGF-beta, Epithelial-Mesenchymal Transition, Hepatocellular Carcinoma

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Abréviations

AIP4/Itch	Athophin Interacting Protein 4
AKT	v-akt murine thymoma viral oncogene homolog 1
AMH	Anti-Müllerian Hormone
ANGPTL4	Angiopoietin-related protein 4
AP1	Protéine activatrice 1
ApoB	Apolipoprotein B
ARFP	Alternative Reading Frame Protein
ATF3	Activating Transcription Factor 3
ATP	Adénosine TriPhosphate
bHLH	basique Helice Loop Helice
BMP	Bone morphogenic protein
CBP	CREB-binding Protein
CCL4	Carbone tetrachloride
CD4	Cluster de différenciation 4
CDK	Cycline Dependant Kinase
CEH	Cellules Étoilées Hépatiques
CHC	Carcinome HépatoCellulaire
PML	ProMyelocytic Leukemia
cPML	Forme cytoplasmique de PML
CTGF	Connective tissue growth factor
DGAT1	DiacylGlycérol AcylTransférase-1
DMN	Dimethylnitrosamine
EGF	Epidermal Growth Factor,
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular signal-regulated kinases
FAK	Focal Adhesion Kinase
FOXC2	Forkhead box C2
FOXO3	Forkhead box O3
GADD34	Growth Arrest and DNA Damage-inducible 45
GCL	Grand Complexe Latent
GDF	Growth and Differenciation Factors
GL	Gouttelettes lipidiques
GTPases	Guanosine TriPhosphatases
HGF	Hepatocyte growth factor
HMGA2	High-mobility group AT-hook 2
HRas	Harvey Rat sarcoma viral oncogene homolog
HVR	HyperVariable Region
IFN	Insulin-like growth factor
IHC	Immunohistochimie
IRES	Internal Ribosome Entry Site
ISDR	Interferon sensitivity-determining region
JFH-1	Japanese Fulminant Hepatitis
JNK	**c**-Jun N-terminal kinase
KLF8	Krueppel-like factor 8

LAP	Latency Associated Protein
LTBP	Latency TGF-β Binding Protein
MAPK	Mitogen activated protein kinase
MEC	Matrice Extra Cellulaire
MIF	Macrophage migration inhibitory factor
MMP	Matrix-metallo proteinase
MSK1	Mitogen- and stress-activated kinase 1
MTMR4	Myotubularin related protein 4
mTor	Mammalian target of rapamycin
MTP	Microsomal triglyceride transfer protein
NAFLD	Non-alcoholic fatty liver
NASH	non alcoholic steato-hepatitis
NC	Non Codant
NT/cNT	Protéine de capside non tumeur
PCL	Petit Complexe Latent
PDGF	Platelet-derived growth factor
PHFRI	PH and Ring Finger domains 1
PI3K	Phosphoinositide 3-kinase
PKR	Protein kinase R
PM1A/PP2Cα	Protéine phosphatase 1A
PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase
Raf	proto-oncogene, serine/threonine Raf-1 kinase
RE	Réticulum Endoplasmique
RhoA	Ras homolog gene family, member A
ROS	Reactive Oxygen Species
SARA	Smad Anchor for Receptor Activation
SBE	Smad Binding Element
Ski	Sloan-Kettering institute
SMA	Smooth Muscle Actin
αSMA	alpha Smooth Muscle Actin
γSMA	gamma Smooth Muscle Actin
Smurf2	Smad ubiquitin related factor2
SNAIL	
SNARK	Sucrose, non-fermenting 1/AMP-activated protein kinase-related kinase
Sno	Ski related novel gene
SP	Signal peptidase
SPP	Signal peptide peptidase
Src	Proto-oncogene tyrosine-protein kinase
SREBP1	Sterol regulatory element binding protein 1
SS	Sequence signal
STRAP	Serine-Threonine kinase Receptor-Associated Protein
T/cT	Protéine de capside isolée de la zone tumorale
TβR1	Récepteur type 1 du TGFβ
TGF-β	Transforming Growth Factor β
TGIF	TG-interacting factor
TIF1γ	Transcriptional Intermediary Factor 1 gamma
TIMP	Tissue inhibitor of matrix-metallo proteinase

TNF	Tumor Necrosis Factor
TRAF	TNF receptor associated factor
TSP-1	Thrombospondine-1
VEGF	Vascular Endothelial Growth Factor
VHB	Virus de l'Hépatite B
VHC	Virus de l'Hépatite C
VLDL	Very low density lipoprotein
WNT1	Wingless-type MMTV integration site family, member 1
YAP-65	Yes-Associated Protein 65

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Introduction

Chapitre I :

Histoire Naturelle de l'infection par le VHC

État des connaissances

I-1. Généralités

Le virus de l'hépatite C (VHC) a été découvert en 1989 à partir de sérum de patients atteints d'hépatite dite non -A non -B (Choo et al, 1989). Le VHC se transmet principalement par voie sanguine (transfusions, utilisation de drogues injectées), mais la contamination par voie sexuelle ou par voie materno-fœtale est possible. Dans le monde, environ 150 millions de personnes sont infectées chroniquement par le VHC et plus de 350 000 individus meurent chaque année de pathologies hépatiques liées à l'hépatite C. Chaque année, on estime que 3 à 4 millions de personnes sont nouvellement infectées par le VHC. La gravité de l'hépatite C est variable. En effet, environ 55 à 85 % des personnes infectées développeront une hépatite chronique qui évoluera vers des maladies hépatiques graves comme la fibrose et le cancer du foie (Fig.1) (Maasoumy & Wedemeyer, 2012; Maillard, 2011) (OMS 2012 - 2014).
A la découverte du VHC, le seul traitement disponible était l'interféron *alpha* qui permettait des taux de guérisons de l'ordre de 10% (Takeda et al, 1993). Au fil des années, le traitement à l'interféron a été amélioré par l'ajout de la ribavirine et a permis de guérir 50% des patients chroniquement infectés par le VHC (Shiffman et al, 2007). En 2011, deux nouvelles molécules font leur apparition le telaprevir et le boceprevir, premiers inhibiteurs directs du VHC (Jensen, 2011). Ces médicaments sont administrés en combinaison au traitement de l'interféron et de la ribavirine. Les taux de guérison de l'infection ont augmenté de 15 à 20%. Ce traitement s'avère efficace uniquement contre un certain type de virus. Cette trithérapie s'accompagne d'effets secondaires très fréquents et parfois très sévères. Après des années de recherche, deux nouveaux médicaments, le simeprevir et le sofosbuvir, des inhibiteurs de protéases du virus font naitre un espoir de guérison complète chez les patients. Le mode d'action de cette bithérapie est de bloquer la multiplication du virus (Lawitz et al, 2014). Les taux de guérison de l'infection dans les études cliniques sont supérieurs à 90% avec peu d'effets secondaires.

I-2. L'hépatite C chronique

L'infection par le VHC est une cause majeure d'hépatite chronique définie par une infection persistante de six mois, une sérologie VHC positive et la présence de l'ARN du virus. Dans la plupart des cas, la maladie est asymptomatique. Son évolution est fonction de l'origine ethnique, de l'âge, du sexe et dépend de nombreux facteurs aggravants comme l'alcool, le

surpoids et le génotype du virus. La physiopathologie de l'hépatite C chronique est liée à l'inflammation induite par la réponse immune dirigée contre les antigènes du virus. De plus, les patients atteints d'hépatite chronique virale C développent dans 40 à 60% des cas une stéatose. Particulièrement, chez les patients infectés par le génotype 3, le degré de stéatose est significativement plus élevé que chez les patients infectés par un autre génotype et significativement corrélé à la concentration intra-hépatique en ARN du VHC, suggérant l'implication directe du VHC dans la genèse de la stéatose chez ces patients. (Rubbia-Brandt et al, 2001; Rubbia-Brandt et al, 2000). La stéatose est associée au risque de progression de la fibrose et de la cirrhose hépatique.

I-2-1. Fibrose et cirrhose

La fibrose hépatique se manifeste par l'accumulation de protéines fibrillaires en excès dans la matrice extra cellulaire (MEC) hépatique. La fibrose liée à l'infection chronique par le VHC est principalement de siège périportal (Bataller & Brenner, 2005). Elle est le résultat de l'augmentation du dépôt et de la production des protéines de la matrice (fibrogenèse) et de la diminution de la dégradation de ces protéines (fibrolyse). Le degré de fibrose est évalué semi-quantitativement par analyse histologique d'un prélèvement hépatique en utilisant le score METAVIR qui définit 4 stades de fibrose (Poynard et al, 1997). La réalisation de biopsies hépatiques successives a permis de mesurer la vitesse de progression de la fibrose en unités METAVIR et de définir des profils de patients fibroseurs lents ou rapides (Poynard et al, 1997). D'autres méthodes non invasives, d'évaluation de la fibrose ont été développées, telles que le Fibrotest et le Fibroscan (Imbert-Bismut et al, 2001) (Ziol et al, 2005). Le stade ultime de la fibrose est la cirrhose. Le délai de développement de la cirrhose est très variable, de 10 à 40 ans après contamination par le virus (Fattovich et al, 1997) (Poynard et al, 2001). Elle se caractérise par la destruction de cellules hépatiques et leur régénération sous forme de nodules de régénération entourés par du tissu fibreux et conduit à la perte des fonctions du foie.

I-2-2. Carcinome Hépatocellulaire

Le stade ultime de la pathologie liée au VHC est le carcinome hépatocellulaire (CHC).
Dans les pays occidentaux, le CHC est la plus fréquente des tumeurs épithéliales primitives du foie ; il se développe le plus souvent sur une cirrhose. L'infection chronique par le VHC fait partie avec le syndrome dysmétabolique et la consommation excessive d'alcool des

principaux facteurs de risque de survenue d'un CHC (Lau & Lai, 2008) (Cabrera & Nelson, 2010). Actuellement, la transplantation apparaît comme l'option thérapeutique optimale car elle conduit non seulement à l'ablation de la tumeur, mais aussi au remplacement de l'organe qui est susceptible d'être le siège d'une nouvelle tumeur (Adam et al, 2003). La transplantation hépatique est soumise à une réglementation, les critères de Milan, elle est recommandée pour les patients dont le foie est relativement fonctionnel avec 2 à 3 tumeurs de petites tailles inférieures à 5 cm. Le taux de survie global à 5 ans après transplantation de ces patients atteint 70% (Veilhan & Adam, 2001). Cependant si le VHC n'est pas éradiqué avant la transplantation, le foie transplanté est rapidement réinfecté. La résection hépatique est une autre option pour le traitement du carcinome hépatocellulaire à un stade précoce mais la récidive à 5 ans est importante (Roudot-Thoraval et al, 1997). Aucune chimiothérapie n'est efficace dans le CHC. A ce jour, la seule thérapie ciblée ayant obtenu une autorisation de mise sur le marché dans le CHC avancé est le sorafénib, un inhibiteur qui cible plusieurs kinases et bloque l'angiogenèse (Rimassa & Santoro, 2009).

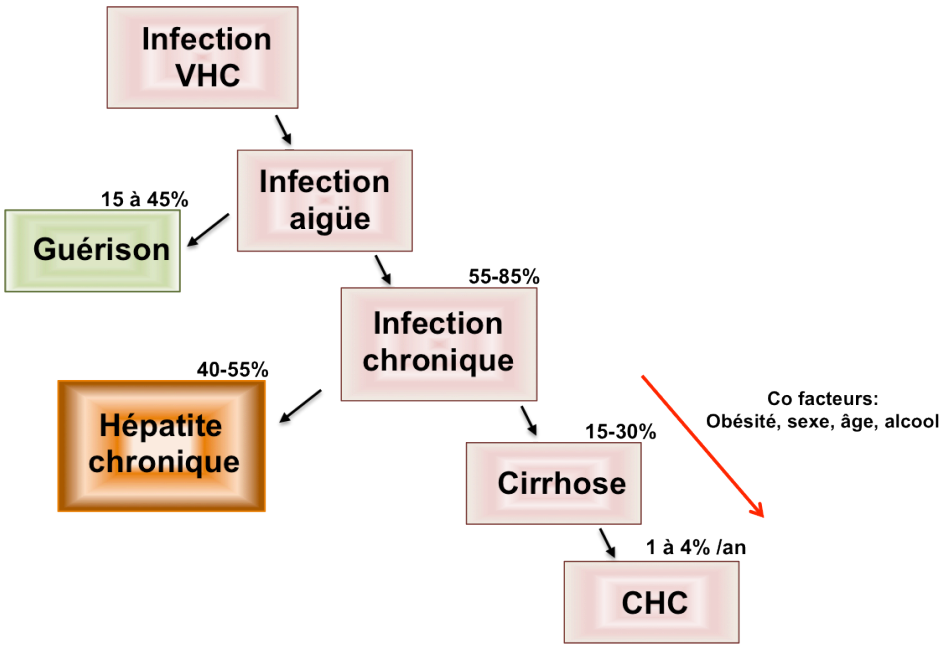

Figure 1: Evolution de l'infection par le VHC

Le virus de l'hépatite C est responsable à la fois de l'infection aiguë et de l'infection chronique. La forme aiguë de la maladie est généralement asymptomatique. La plupart des patients atteints par le VHC développent une infection chronique. Pour la majorité l'infection évoluera vers la forme chronique de la maladie. Parmi celle-ci, le risque de cirrhose du foie est de 15 à 30% sur une durée de 20 ans.

I-3. Biologie du VHC

I-3-1. Structure et Organisation du Génome du VHC

Le génome du VHC est composé d'un ARN monocaténaire de polarité positive d'environ 9600 pb qui se divise en 3 parties : la région 5'non codante (NC), la phase de lecture (ORF), la région 3' non codante (NC). Les régions 5' NC et 3'NC sont des régions fortement structurées. La région 5', contient la structure cis-acting ARN qui constitue l'IRES et détermine le début de la traduction de la poly-protéine par le ribosome. Outre, son rôle primordial dans l'initiation de la traduction de l'ARN viral du VHC, l'IRES est également nécessaire pour la réplication de l'ARN. La phase de lecture ouverte unique de l'ARN du VHC code pour une poly-protéine d'environ 3000 acides aminés.

I-3-2.Les protéines du VHC

La phase de lecture ouverte code pour une poly-protéine qui est clivée en dix protéines par des protéases virales et cellulaires, dont la séquence est la suivante NH2-Capside-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Ces protéines sont deux types de protéines :

- les protéines structurales : capside, deux glycoprotéines d'enveloppe (E1 et E2). Ces protéines sont libérées à partir de la poly-protéine virale par les protéases cellulaires peptidases et peptide peptidase.
- les protéines non structurales : P7, NS2, NS3, NS4A, NS4B, NS5A et NS5B qui assurent les fonctions enzymatiques nécessaires au cycle viral. Après libération des protéines structurales de la poly-protéine, c'est la protéase virale qui permet la production des différentes protéines non structurales (Fig.2). Une onzième protéine est décrite, appelée protéine F (ARFP, Alternate Reading Frame Protein), elle est le résultat du décalage du cadre de lecture de la région codant pour la capside.

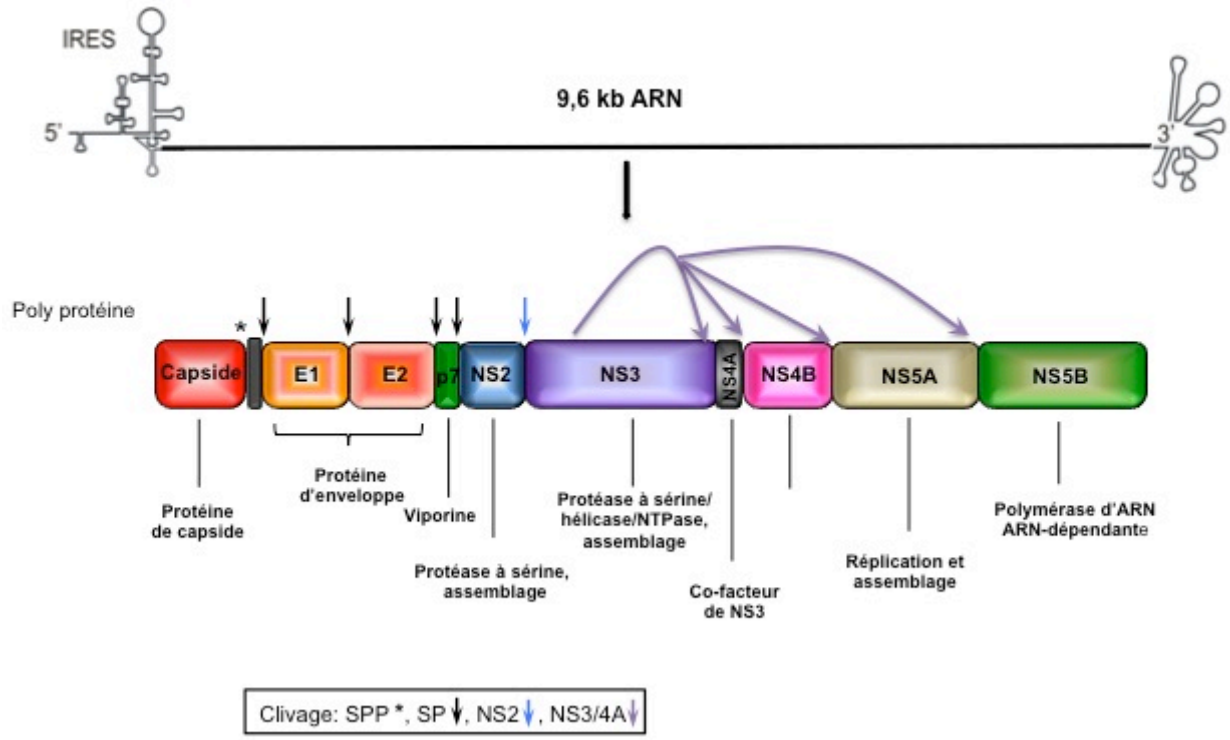

Figure 2 : Schéma du génome et de la poly-protéine du VHC

Le génome encode une poly-protéine qui est flanquée par les régions 5' et 3' non codantes. La phase de lecture de l'ARN messager du VHC code pour une poly-protéine clivée en 3 protéines structurales, Capside, E1, E2, et de 7 protéines non structurales, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B. La protéine NS2 est responsable du clivage des protéines NS2/NS3, tandis que le complexe NS3/4A clive les autres complexes indiqués par les flèches. Schéma adapté (Fusco & Chung, 2012)

I-3-2-1. Protéines structurales

La protéine de capside

La protéine de capside est l'une des premières protéines traduites et forme la nucléocapside virale entourant l'ARN du virus. Elle est d'abord clivée par un signal peptidase (SP) cellulaire au niveau d'une séquence signal (SS) située en C-terminal. Ce clivage libère la forme immature de la protéine basique de 191 aa, soit 21 kDa ancrée par le SS au RE (McLauchlan, 2000). Le deuxième clivage protéolytique se produit au niveau du peptide signal *via* une signal peptide peptidase (SPP) (Hussy et al, 1996), donnant lieu à une forme plus courte, de 173 aa, qui correspond à la protéine mature de la capside de 19 kDa, localisée majoritairement dans le cytoplasme (Moradpour et al, 2007). La protéine de capside est organisée de trois domaines : le domaine D1 en N-terminal est constitué de 117 aa de nature hydrophile et chargé négativement, le domaine D2, des aa 118 à 173 de nature hydrophobe interfère avec le métabolisme lipidique (McLauchlan, 2000), le domaine D3, des aa 175 à

191 constitue un peptide signal pour le clivage entre la capside et E1 (Hope & McLauchlan, 2000). Enfin, certaines interactions de la protéine de capside avec les protéines cellulaires de l'hôte sont cruciales pour le repliement et la stabilité de la protéine (Boulant et al, 2005) (Boulant et al, 2006). La protéine de capside interagit avec de nombreuses protéines cellulaires modulant ainsi la signalisation cellulaire, la transcription de gènes, la prolifération et l'apoptose cellulaires (McLauchlan, 2000);(Tellinghuisen & Rice, 2002). Ces fonctions seront développées dans un paragraphe particulier (chap. IV.4).

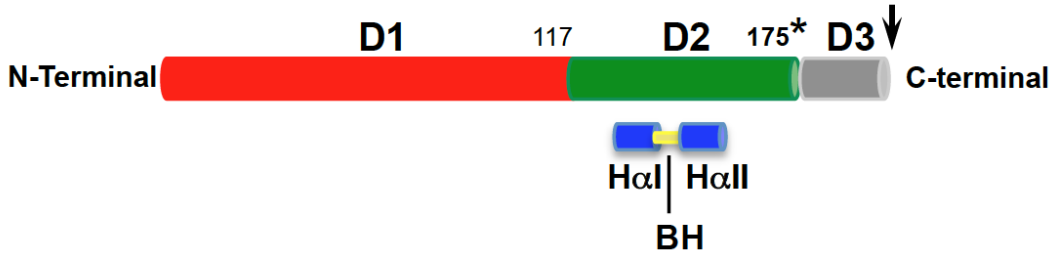

Figure 3: Structure de la protéine de capside du VHC

La capside mature peut être séparée en trois domaines. D1 est impliqué dans les interactions protéine-protéine et se lie l'ARN. D2 contient une structure double hélices α (HI et HII) séparées par une boucle hydrophobe (BL) qui intervient dans la conformation et la stabilité de D1. Cette structure HI-HL-HII est impliquée dans la localisation à la surface des gouttelettes lipidiques. D3 renferme les sites de clivage pour le signal peptide peptidase (*) et le signal peptidase (flèche noire). Figure adapté (McLauchlan, 2009).

Les glycoprotéines d'enveloppe E1-E2

Les glycoprotéines d'enveloppe E1 et E2 sont clivées par une signal peptidase cellulaire au niveau de la membrane du RE (Penin et al, 2004). Ces glycoprotéines d'enveloppe sont des protéines membranaires de type I comportant toutes deux un ectodomaine N-terminal de 160 et 334 aa respectivement, et un domaine transmembranaire C-terminal d'une trentaine d'aa (Cocquerel et al, 2000). Les domaines transmembranaires ont une double fonction : ils permettent d'une part l'ancrage des glycoprotéines dans la membrane du RE et, d'autre part, ils jouent un rôle important dans la formation des hétérodimères E1/E2 non-covalents (Penin et al, 2004). Les deux glycoprotéines d'enveloppe, sont essentielles à l'attachement aux récepteurs cellulaires et à l'entrée du virus en induisant la fusion de la membrane de la cellule hôte. De plus, la glycoprotéine E2 joue également un rôle central dans l'échappement viral *via* l'interaction avec CD81. En effet, *in vitro*, cette interaction inhibe l'activation des lymphocytes T et des cellules NK (Natural killer) et permet ainsi l'établissement de l'infection chronique (Crotta et al, 2002 ; Tseng & Klimpel, 2002).

La protéine F ou ARFP

La protéine F est une protéine de 16-17 kDa, nommée F (frameshift) ou ARFP (alternate reading frame protein) (Walewski et al, 2001) (Xu et al, 2001). Elle est générée par un changement de cadre de lecture alternatif chevauchant la séquence de la capside. L'initiation de la traduction de la protéine F se fait au niveau du codon AUG de l'ARN suivie d'un glissement ribosomal -2/+1 au codon 8-11 dans la région amino-terminal (Walewski et al, 2001) (Xu et al, 2001). L'expression de la protéine n'a jamais pu être observée dans les tissus ou le sérum de patient. Mais l'existence de cette dernière a été détectée par la présence d'anticorps spécifiques dans le sérum de patients infectés par le VHC quelque soit le génotype du virus (Budkowska et al, 2011), (Cohen et al, 2007), (Walewski et al, 2002), (Walewski et al, 2001), (Xu et al, 2001). Cette protéine est unique et ne présente aucune homologie avec d'autres protéines virales connues. Par ailleurs, des travaux suggèrent que la synthèse de la protéine F n'est pas nécessaire à la réplication du VHC *in vitro* (McMullan et al, 2007) mais participerait à l'infectiosité en facilitant l'assemblage et la libération des virions (Steinmann et al, 2007) (Ratinier et al, 2009),

I-3-2-2. Protéines non structurales

La protéine p7

La protéine p7 est une protéine membranaire de 63 aa, contenant deux domaines transmembranaires qui l'orientent vers la lumière du RE. Du fait de ces caractéristiques, la protéine p7 est classée dans la famille des protéines viroporines. Des données *in vivo*, ont montré que la protéine p7 était nécessaire à la réplication du virus (Sakai et al, 2003). Elle serait également impliquée dans le relargage des particules virales par la fonction d'un canal ionique (Steinmann et al, 2007). De plus, un des rôles de la protéine p7 serait d'équilibrer le pH dans les compartiments cellulaires afin de rendre possible la production de particules virales pendant le processus de maturation (Wozniak et al, 2010). Une étude récente montre que la protéine p7 est requise dans les étapes ultimes d'assemblement et d'enveloppement du génome viral (Gentzsch et al, 2013). L'ensemble de ces découvertes témoigne que la protéine p7 jouerait un rôle majeur dans les étapes tardives du cycle viral, durant l'assemblage et la sécrétion des particules virales infectieuses (Bentham et al, 2013).

Protéine NS2

La protéine NS2 est une protéine transmembranaire non glycosylée de 23 kDa. Elle comporte dans la région N-terminale des résidus d'aa hautement hydrophobes formant trois domaines trans-membranaires associés au RE. Le domaine C-terminal de la protéine NS2 est lié au domaine N-terminal de la protéine NS3 conférant ainsi la qualité auto-protéase à NS2 responsable de leur clivage (Schregel et al, 2009). Il a été montré que le clivage de ces deux protéines est indispensable pour la réplication d'ARN et pour le cycle viral (Gorzin et al, 2012).

Protéine NS3 -4A

NS3 est une protéine bi-fonctionnelle de 70 kDa qui possède une activité serine protéase localisée dans le premier tiers de la région N-terminale et une activité ARN hélicase / NTPase localisée dans les deux tiers de la région terminale (Tai et al, 1996). NS4A est une petite protéine trans-membranaire de 8 kDa qui interagit *via* son domaine C-terminal avec les membranes et d'autres composants de la réplication. Elle forme avec la protéine NS3 un complexe nécessaire pour le clivage de la poly-protéine et la réplication virale. En effet, l'activité protéase NS3/4A, est responsable du clivage des jonctions NS3/4A, NS4A/NS4B, NS4B/NS5A et NS5A/NS5B. Cette activité est indispensable à la formation du complexe de réplication. Outre son rôle dans le cycle viral, NS3/4A interfère avec plusieurs voies de la signalisation cellulaire intervenant ainsi dans la carcinogenèse et dans l'échappement du virus à la réponse immunitaire (Pang et al, 2002) (Dubuisson, 2007) (Suzuki et al, 2007).

De plus, l'activité ARN hélicase permet de séparer les doubles brins de l'ARN et de faciliter l'accès de l'ARN polymérase virale NS5B, aux structures très repliées, comme l'IRES en 5'NC en hydrolysant l'ATP. Elle permet à la fois l'ancrage et la stabilisation de NS3 à la membrane du RE en lui évitant ainsi une dégradation protéolytique.

Protéine NS4 B

NS4B est une protéine hydrophobe transmembranaire d'environ 30 kDa. Sa fonction reste encore mal connue, mais elle est associée aux autres protéines non structurales dans le complexe de réplication virale (membranous web) (Bartenschlager & Lohmann, 2000). Sa modification post traductionelle (palmitoylation) est essentielle pour la réplication virale, (Krekulova et al, 2006).

Protéine NS5 A

La protéine NS5A est une phosphoprotéine associée aux membranes cellulaires. On retrouve deux formes de cette protéine, soit 56 kDa ou 58 kDA, correspondant aux formes phosphorylées. La structure 3D de cette protéine a permis de mettre en évidence trois domaines numérotés de I à III séparés par des séquences de faibles complexités (low complexity sequence ou LCS) (Tellinghuisen et al, 2004). Le domaine I contient au niveau de la région N-terminale l'hélice-α amphipathique, responsable de l'association de NS5A à la membrane et un motif d'attachement au zinc. Ce domaine permet l'interaction protéine-protéine essentielle à la formation et la fonction du complexe de réplication du génome viral (Penin et al, 2004) (Tellinghuisen et al, 2005). Le domaine II, contient une région appelée ISDR (interferon sensitivity determining region) (Tellinghuisen et al, 2004). L'ISDR est impliquée dans l'interaction avec la protéine cellulaire PKR induite en réponse à l'IFN (interferon) (Gale et al, 1998). L'interaction entre ISDR et PKR inhibe l'activité antivirale de cette dernière. Le domaine III est une des régions les moins conservées du génome du VHC (Tellinghuisen et al, 2004). Cependant, ce domaine est crucial pour l'assemblage des particules virales. En effet, la perte du domaine III de NS5A perturbe la formation des particules virales infectieuses car elle empêcherait la co-localisation de la protéine de capside avec NS5A au niveau des GL (Appel et al, 2008). De plus l'interaction de NS5A avec l'apolipoprotéine cellulaire ApoE est indispensable pour l'assemblage des particules virales et la libération des virions infectieux (Benga et al, 2010).

Protéine NS5 B

La protéine NS5B est une protéine hydrophile de 65 kDa, qui contient un motif GDD conservé (résidus glycine – acide aspartique – acide aspartique) caractéristique des ARN polymérases virales ARN-dépendantes. La région C-terminale de NS5B forme un domaine transmembranaire α-hélicoïdal qui est responsable de son ancrage dans la membrane du RE et de son orientation cytoplasmique (Moradpour & Blum, 2004). Cette protéine est un acteur majeur pour la réplication du génome viral. En effet, elle assure la synthèse des deux brins complémentaires. Le brin négatif complémentaire, intermédiaire de réplication, sert ensuite de matrice à la synthèse de grandes quantités d'ARN positif. Sa fonction au sein du complexe de réplication en fait une cible majeure pour les traitements antiviraux.

I-3-3. Cycle viral

Depuis la découverte du virus de l'hépatite C, des techniques de biologie moléculaire ont permis l'étude de protéines codées par le génome viral, mais son cycle de réplication est resté longtemps mal connu en raison de l'absence d'un modèle cellulaire permettant la production de particules virales infectieuses. Des modèles de culture cellulaire ont été développés pour étudier la réplication du VHC. Le modèle de « réplicon subgénomique » du VHC a été développé à partir d'un ARN subgénomique du VHC (Lohmann et al, 1999). Ce réplicon est constitué par un génome viral où les protéines structurales ont été supprimées et remplacées par un gène de résistance à la néomycine. Mais ce modèle de réplicon subgénomique a aussi ces limites car il ne permet pas former des particules virales. Des progrès dans la connaissance du cycle infectieux du VHC ont été réalisés grâce au développement d'un modèle de cellules Huh7.5 infectées par un isolat de génotype 2a (souche JFH-1) provenant d'un patient japonais atteint d'une hépatite fulminante (Kato et al, 2001), (Wakita et al, 2005),(Lindenbach et al, 2005), (Burlone & Budkowska, 2009). L'infection d'hépatocytes primaires humains par des particules virales obtenues après une culture cellulaire du modèle JFH-1 permet d'obtenir des particules associées aux VLDL identiques à celles retrouvées dans le sang de patients infectés ce qui permet d'étudier les interactions entre le virus et le métabolisme de la cellule hôte (Podevin et al, 2010).

I-3-3-1. Entrée du virus

L'entrée du VHC dans les cellules est un processus complexe. Les particules virales se lient à la cellule hôte par des interactions spécifiques avec plusieurs récepteurs de surface parmi lesquels le LDL-récepteur, la tétraspanine CD81, le scavenger récepteur Class B SR-B1, la claudine1 ou encore l'occludine. Plus récemment il a été montré que le récepteur au facteur de croissance épithélial, l'EGFR était un co-récepteur impliqué dans l'entrée du virus (Lupberger et al, 2011). En fait, les voies de signalisation de l'EGFR et en particulier la protéine HRas permettent l'assemblage des différents facteurs nécessaires à l'entrée du virus (Zona et al, 2013).

I-3-3-2. Réplication virale

Après la reconnaissance de la particule virale par son récepteur, l'acidification de l'endosome induit un changement de conformation de l'enveloppe virale, la décapsidation de la particule virale, et la libération de l'ARN positif du génome viral. L'ARN polymérase synthétise un brin d'ARN négatif à partir de l'ARN positif. Cet ANR négatif sert de matrice pour la

synthèse de nombreux brins d'ARN positifs. Le génome viral est ensuite traduit au niveau du réticulum endoplasmique en une poly-protéine qui sera clivée par des protéases cellulaires et virales. Une altération de la membrane du réticulum endoplasmique est induite par le virus, sous forme de vésicules qui permettent de concentrer l'ensemble des protéines virales et cellulaires nécessaires à la réplication (Joyce & Tyrrell, 2010). Ce réarrangement est appelé « membranous web » Les ARN (+) seront internalisés dans les particules virales néoformées, composées des protéines de capside de E1 et E2. Les néo-particules s'assemblent dans la lumière du réticulum endoplasmique en association avec les VLDL avant d'être sécrétées dans le milieu extracellulaire (Fig.4) (Icard et al, 2009).

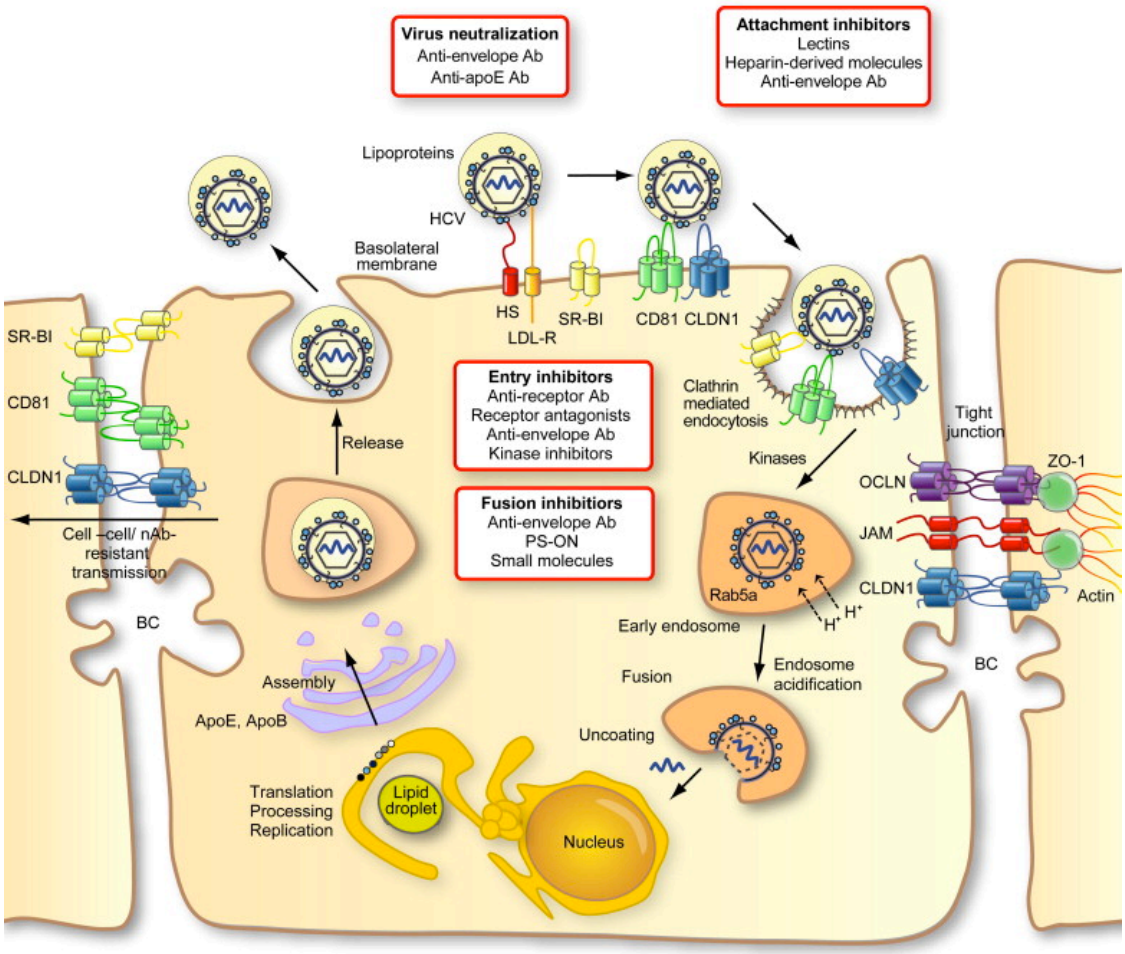

Figure 4: Cycle viral du VHC :

Modèle du cycle viral du VHC: le VHC interagit avec l' HS et le LDL-R à la surface de la membrane basolatérale des hépatocytes et permet la concentration du virion. L'interaction se poursuit avec d'autres facteurs de l'hôte tels que SR-BI, CD81, CLDN1, et OCLN qui conduit finalement à l'internalisation virale par endocytose. La fusion des membranes virales avec celles de l'endosome, suivie par la libération du génome viral dans le cytosol où la traduction et la réplication ont lieu. Des particules du VHC sont ensuite assemblées et libérées de la cellule hôte (Zeisel et al, 2011).

I-3-4. Variabilité génomique

Le virus de l'hépatite C présente une variabilité génétique importante, en raison de l'absence d'activité correctrice exonucléasique 5'→3' de l'ARN polymérase virale (NS5B) qui entraîne un taux d'erreur d'environ 10^{-3} substitutions nucléotidiques par site et par an (Ogata et al, 1991) ; (Okamoto et al, 1992). Le niveau élevé de réplication du virus (10^{12} particules virales au cours d'une hépatite chronique) associé à une absence de correction des erreurs de transcription entraîne l'émergence de nombreux variants viraux. L'analyse phylogénétique des séquences de différentes souches du VHC isolées dans différentes zones géographiques a permis d'identifier 7 génotypes, numérotés de 1 à 7 décomposés en sous-type désignés par une lettre minuscule après la numérotation du génotype (Fig.5) (Kuiken & Simmonds, 2009). Les différents génotypes et sous-types, ne sont pas uniformément répartis dans le monde. Les génotypes 1a, 1b, 2a, 2b, 2c et 3a, sont présents dans les pays industrialisés, avec une prédominance pour le génotype 1a, 1b en Europe et aux USA et le génotype 1b présente une prévalence proche des 100% au Japon. Le génotype 3a, présente une prévalence plus faible aux Etats-unis qu'en Europe. Le génotype 4 est surtout représenté au Moyen-Orient et en Afrique du Nord, le génotype 5 est présent uniquement en Afrique du Sud , le génotype 6 est surtout localisé en Asie et enfin le génotype 7 aurait son origine dans le centre Afrique (Zein, 2000).

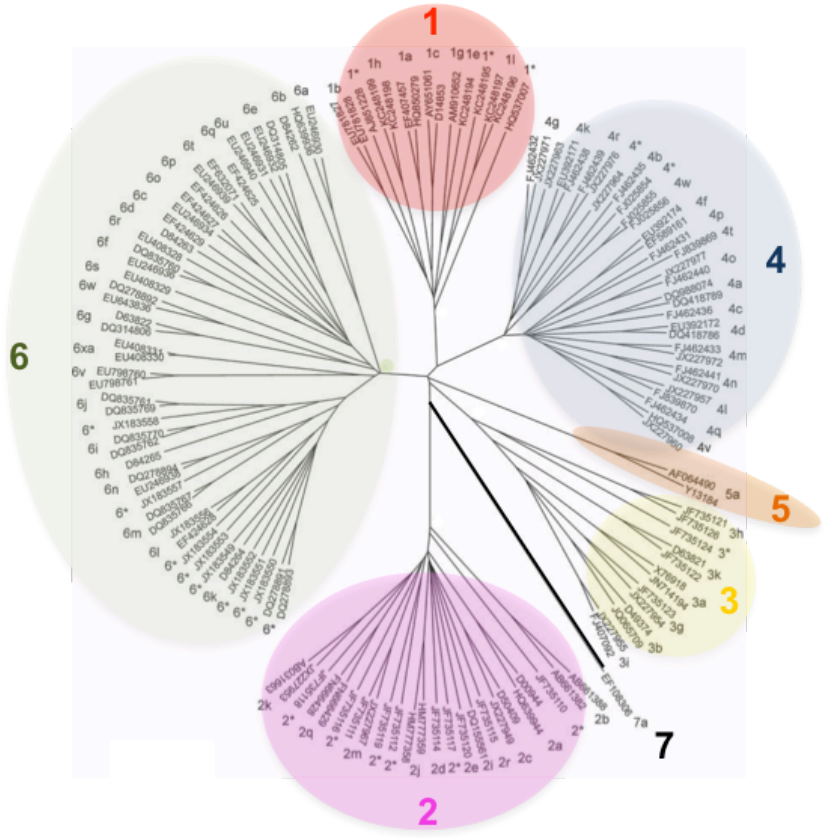

Figure 5 : Variabilité du VHC

Arbre phylogénique représentant les 7 génotypes et les différents sous-types du VHC Schéma adapté (Smith et al, 2014)

La variabilité génomique du VHC est influencée à la fois par des pressions immunitaires de l'organisme hôte et par la présence de zones très variables reparties de façon hétérogène sur le génome du VHC. En effet, il existe 2 types de mutations, qui sont dites:

- Silencieuses / synonymes, (elles n'ont aucun impact sur la structure en aa mais vont pouvoir modifier la structure secondaire de l'ARN génomique).
- Non synonymes (elles ont un impact sur la structure de la protéine et provoquent ainsi l'apparition de nouveaux variants).

Le génome du VHC comporte aussi des régions bien conservées correspondant à des fonctions indispensables pour le cycle viral comme la réplication et la traduction. En effet, la région 5'NC fait partie des régions du génome les mieux conservées avec une homologie de séquence entre diverses souches du VHC estimée à plus de 90% (Bukh et al, 1992). Il en est de même avec le gène qui code pour la protéine de capside qui présente une similitude de séquence nucléotidiques estimée entre 79,4% et 99% (Bukh et al, 1994). En revanche, les

régions codantes pour les glycoprotéines d'enveloppes E1 et E2 sont les parties du virus les plus variables. Notamment, les régions HVR1, HVR2, HVR3 de la protéine E2 et la région C-terminale de la protéine NS5A présentent plus de 50% de variations nucléotidiques entre les différentes souches du VHC (Pawlotsky, 2003).

1-3-4-1.Quasi-espèces

Pendant l'évolution de la maladie, de nouveaux variants viraux apparaissent chez le patient, issus du même génotype mais génétiquement distincts, appelés quasi-espèces. La quasi-espèce comprend un ou plusieurs variants majoritaires et une multitude d'autres variants différents présentant une homologie de séquence de 90% (Pawlotsky, 2003). En circulant sous forme de quasi-espèces, le virus assure sa persistance virale en sélectionnant des variants résistants à leur environnement immunologique au cours de l'infection. Les traitements antiviraux exercent aussi des pressions plus ou moins fortes induisant des changements au sein de la quasi-espèce (Sakai et al, 1999; Shimizu et al, 1997).

Chapitre II

La biologie du TGF-β

2-1. La superfamille des ligands TGF-β

Généralité

Le TGF-β (Transforming Growth Factor Beta 1) est une cytokine multifonctionnelle, secrétée par un grand nombre de types cellulaires. Le TGF-β joue un rôle important dans le développement de l'embryon dans la régulation des réponses cellulaires par ses différentes actions sur la prolifération, la différenciation, l'apoptose, le développement et le remodelage de la matrice extracellulaire (MEC) et la régulation de l'homéostasie des lymphocytes (Rahimi & Leof, 2007), (Levy & Hill, 2006) (Yue & Mulder, 2001). Ses effets varient selon le type cellulaire et l'état physiologique de la cellule. En effet, le TGF-β est capable d'inhiber la prolifération des cellules épithéliales, endothéliales et hématopoïétiques, et de réguler la différenciation cellulaire, d'induire l'apoptose de ces cellules et au contraire de provoquer la prolifération des fibroblastes. Les nombreux effets du TGF-β et sa répartition ubiquitaire dans l'organisme expliquent son implication dans diverses pathologies. Le TGF-β est considéré comme un acteur principal de la fibrose car il augmente l'expression des composants de la matrice extracellulaire (MEC). Un nombre considérable d'études ont montré un rôle majeur du TGF-β dans les mécanismes de la cancérogenèse. Si, dans les étapes précoces de la cancérogenèse, le TGF-β agit comme un suppresseur de tumeur en inhibant la croissance cellulaire et en favorisant l'apoptose, il intervient, dans les étapes plus avancées du processus, comme un promoteur de tumeur. En effet, les cellules cancéreuses sécrètent du TGF-β en grande quantité, alors qu'elles deviennent résistantes à ses effets anti-prolifératif et apoptotique (Wakefield & Roberts, 2002). Dans ces cellules, le TGF-β est toujours capable d'induire une transition épithélio-mésenchymateuse (TEM) favorisant ainsi le processus de migration et d'invasion (Oft et al, 1998). (La TEM sera détaillée dans le chapitre III).

Le TGF-β

Le TGF-β fait partie d'une superfamille divisée en plusieurs sous-familles qui présentent des similitudes fonctionnelles et structurales. Cette superfamille comprend les isoformes du TGF-β, les activines, la protéine Nodal, les protéines BMP (bone morphogenetic proteins) et les GDF (growth and differentiation factors) (Fig.6) (van Dijk et al, 2012). Chez l'homme, on compte 3 isoformes du TGF-β, TGF-β1, TGF-β2 et TGF-β3, codées par trois gènes distincts, présentant une homologie de séquences protéiques qui varie de 71 à 80% (Yue & Mulder,

2001). Ces 3 isoformes ne sont pas redondantes puisque l'invalidation d'un seul gène est létale chez la souris (TGF-β2 ou TGF-β3). Les souris invalidées pour TGF-β1 meurent rapidement d'inflammation massive (Kulkarni & Karlsson, 1993 ; Sanford et al, 1997).

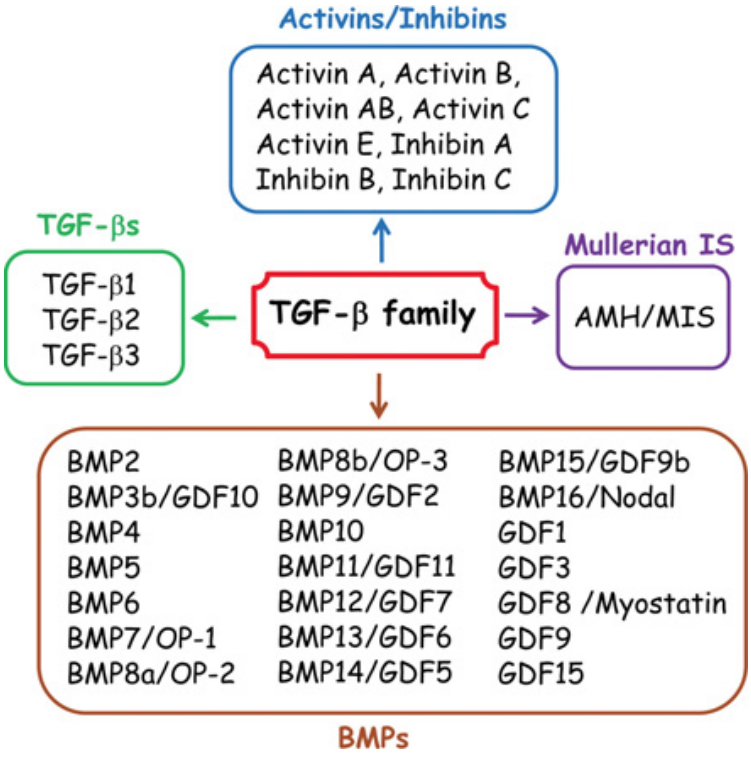

Figure 6 : Membre de la Superfamille TGF-β (Santibanez et al, 2011)

Représentation des 4 groupes des membres de la superfamille TGF-β incluant les TGF-β, les BMP/GDF, AMH/MIF et activine/inhibine

2-2. Synthèse et Activation du TGF-β

2-2-1. Synthèse du Pré-pro- TGF-β

Le TGF-β est sécrété sous la forme d'un grand complexe latent (GCL) qui comporte le petit complexe latent lié à une protéine de la matrice cellulaire LTBP (Latent TGF-β Binding Protein) (Dallas et al, 2006) ;(Miyazono et al, 1988) ; (Wakefield et al, 1988). La protéine LTBP permet le stockage du TGF-β dans la matrice cellulaire. Le petit complexe latent (PCL) est composé d'une protéine homodimérique LAP (latency associated peptide) et du TGF-β mature. La LAP confère la latence au TGF-β en l'empêchant de se lier à ses récepteurs spécifiques (Fig.7) (Moustakas & Heldin, 2009).

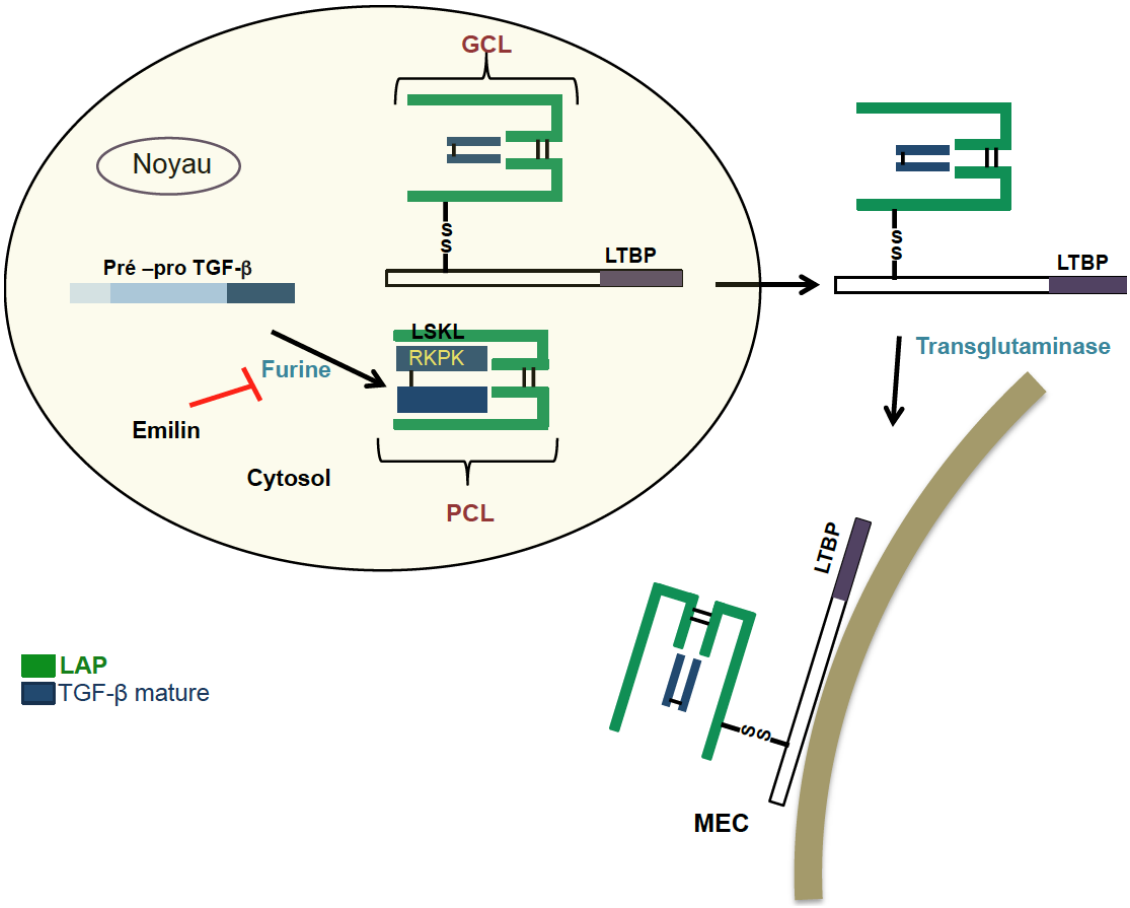

Figure 7 : Synthèse du TGF-β

Le TGF-β est synthétisé sous la forme d'une molécule précurseur le pré-pro TGF-β dans l'appareil de Golgi, Ce précurseur comprend une séquence peptide signal au niveau du domaine N-terminal qui sera la cible du clivage d'une furine convertase. Ce clivage permet la formation du dimère TGF-β mature qui se lie à une protéine homodimérique LAP par un pont disulfure, pour former le petit complexe latent PCL (Petit Complexe Latent). De plus la liaison de la séquence LSKL de la LAP avec la séquence RKPK du TGF-β mature est nécessaire pour le maintien de la latence. Le petit complexe latent se lie par un pont disulfure à une protéine de la matrice cellulaire appartenant à la famille des LTBP (Latent TGF-β Binding Protein). Ce grand complexe latent appelé GCL permet de stocker le TGF-β à la matrice extra-cellulaire (MEC).

2-2-2. Activation du TGF-β latent

L'activation du TGF-β est d'une importance capitale dans les effets biologiques de cette cytokine puisque le TGF-β requiert cette maturation pour se fixer sur son récepteur et induire une réponse cellulaire. Cette activation est très complexe et fait intervenir plusieurs processus comme la protéolyse, des changements conformationnels de la LAP par liaison avec des protéines cellulaires comme les intégrines ou la thrombospondine 1 (Hayashi & Sakai, 2012), les ROS (Reactive Oxygen species) (Barcellos-Hoff & Dix, 1996) et des acidifications du milieu (Jullien et al, 1989), afin de libérer le TGF-β mature de la LAP et le rendre actif.

Thrombospondine-1:

La TSP-1 a été découverte comme une protéine libérée des granules α des plaquettes (Lawler et al, 1978). Elle est sécrétée par une variété de cellules dont les plaquettes, les fibroblastes et les cellules endothéliales. Elle participe à l'agrégation des plaquettes et à des processus fondamentaux de prolifération, d'adhérence, de migration cellulaire, d'embryogenèse, d'angiogenèse et de reconstruction de la MEC (Bornstein, 2009). Elle est également un acteur crucial dans l'activation du TGF-β (Schultz-Cherry et al, 1994). La TSP-1 est une glycoprotéine trimérique de la MEC. Chaque sous-unité comporte des extrémités amino - et carboxy - terminales et une région centrale présentant une homologie à la chaine 1 du pro collagène de type 1 et des motifs répétés de type I (TSP- like), type II (EGF-like) et de type III (liant le calcium) (Fig.8). Deux motifs peptidiques situés dans le domaine de *répétition de type* I sont nécessaires à l'activation du TGF-β, la séquence KRFK interagit avec la séquence LSKL du pro-peptide amino-terminal de la LAP du TGF-β latent, qui conduit à la dissociation du complexe LAP/TGF-β (Schultz-Cherry et al, 1995; Schultz-Cherry et al, 1994). Le second motif nécessaire à l'activation du TGF-β est présent sur chacun des domaines *répétition type I*. Il s'agit de trois peptides de 4 acides aminés identifiés WxxW (WSPW, WSHW et WGPW) qui lient un site actif situé à l'extrémité C-terminale du TGF-β mature. La séquence WxxW stabilise le complexe TGF-β/TSP-1 de façon à orienter RFK et facilite ainsi la liaison de la séquence avec son site de liaison LSKL de la LAP (Murphy-Ullrich & Poczatek, 2000). L'activation du TGF-β résulte d'un changement de conformation de la LAP qui libère le TGF-β actif (fig.9). Des mutations dans la séquence LSKL de la LAP sont suffisantes pour inhiber l'activation du TGF-β par la TSP-1 (Young & Murphy-Ullrich, 2004). De même, l'utilisation d'un peptide inhibiteur LSKL va empêcher la liaison de la TSP-1 à la LAP et bloquer ainsi l'activation du TGF-β (Crawford et al, 1998). En effet, l'utilisation de l'inhibiteur LSKL dans un modèle de fibrose hépatique induit par un traitement au dimethylnitrosamine (DMN) chez le rat montre une réduction de l'activité du TGF-β locale et supprime ainsi la progression de la fibrose (Kondou et al, 2003). Il a été mis en évidence chez des souris transgéniques déficientes pour la TSP-1 que la perte de l'expression de la TSP-1 diminue les réponses biologiques du TGF-β en terme de prolifération cellulaire (Hayashi et al, 2012)

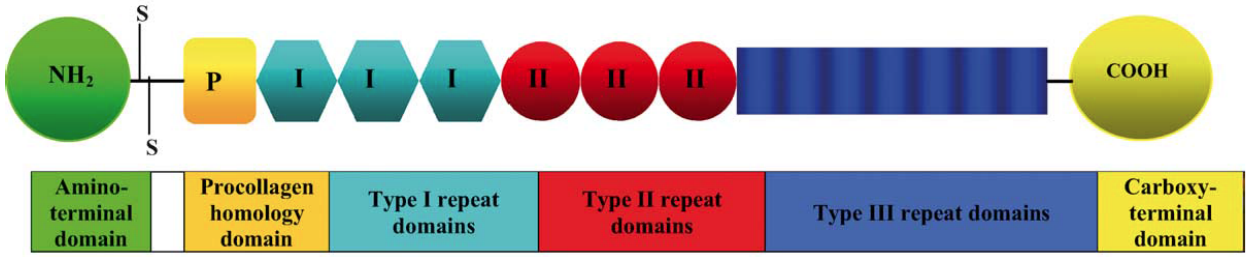

Figure 8 : Représentation schématique de la structure de TSP-1 (Sid et al, 2004)

La thrombospondine est une molécule composée de 3 chaines de poids moléculaires identiques reliées par des ponts disulfures. Chaque sous-unité présente un domaine N-terminal et C-terminal globulaires encadrant une longue région linéaire présentant une homologie à la chaine 1 du pro collagène de type 1 et des motifs répétés de type I (TSP- like), type II (EGF-like) et de type III (liant le calcium).

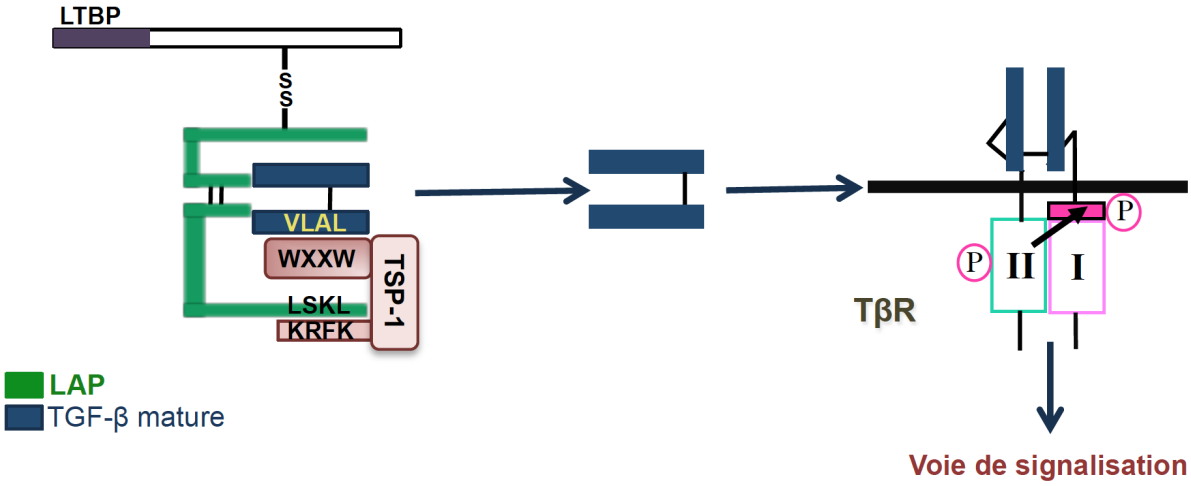

Figure 9 : Activation du TGF- β latent par la thrombospondine

Deux séquences présentes dans le domaine Type 1 de la TSP-1 sont nécessaires à l'activation du TGF-β latent. Une séquence de 3 aa (RFK) est présente sur chacun des « type 1 repeat » de la TSP-1. Une autre séquence de 4 aa WxxW lie la séquence VLAL situé à l'extrémité c-ter du TGF-β mature. Cette séquence a pour rôle de stabiliser le complexe TGF-β/TSP-1 de façon à orienter KRFK et ainsi faciliter l'interaction avec la LAP. Cette interaction permet l'activation du TGF-β latent par un changement conformationnel. Le TGF-β peut ensuite se lier aux récepteurs et induire une signalisation.

Intégrines

Les intégrines sont des molécules hétérodimériques, impliquées dans l'adhésion cellulaire et l'activation de récepteurs membranaires. Elles sont composées de deux sous-unités α et β, comportant un large domaine extracellulaire et un court domaine cytoplasmique (van der Flier & Sonnenberg, 2001). Des travaux témoignent que les intégrines peuvent réguler l'activation *in vivo* du TGF-β latent (Yang et al, 2007) ;(Munger et al, 1999). En particulier,

deux intégrines sont connues pour à la fois interagir et activer le TGF-β latent : αVβ6 et αVβ8 (Araya et al, 2006) ; (Annes et al, 2002) ;(Munger et al, 1999). La sous-unité α contient une séquence RGD qui permet une liaison directe avec la LAP mais n'est pas suffisante pour l'activer. Ainsi, la région cytoplasmique de la sous-unité β interagit avec le cytosquelette d'actine de la cellule et induit une traction commune des sous-unités qui permet la libération du TGF-β actif (Munger et al, 1999). Des études *in vivo,* montrent l'importance de la sous unité β6 dans l'activation du TGF-β latent car son absence est associée à une baisse de la signalisation du TGF-β (Wang et al, 2007).

Protéolyses :

De nombreuses protéases, comme la plasmine, les métalloprotéines (MMP)-2/9 ont été identifiées *in vitro,* comme capables d'activer le TGF-β latent (Yu & Stamenkovic, 2000) ; (Sato & Rifkin, 1989) ; (Lyons et al, 1988). Les protéases peuvent agir selon trois modes d'action :

Soit en induisant un clivage de la protéine LTPB ce qui permet la libération du TGF-β actif du PCL (Taipale et al, 1994), soit par un clivage de la protéine LAP entraînant ainsi la déstabilisation des interactions LAP-TGF-β (Lyons et al, 1988), ou comme la protéase ADAMST1, en se liant à la LAP ce qui induit un changement conformationnel libérant le TGF-β actif (Bourd-Boittin et al, 2011).

2-3- La Voie de signalisation canonique du TGF-β

2-3-1.Famille des TGF récepteurs

Pour transmettre une réponse biologique, les ligands de la famille TGF-β se fixent sur des récepteurs spécifiques contenant un domaine sérine/thréonine. Il existe 3 types de récepteurs membranaires : les récepteurs de type I, TβRI, de type II, TβRII et de type 3 TβRIII appelés récepteurs accessoires. Les différents membres de la famille du TGF-β activent plusieurs voies de signalisations, cependant la voie de transduction principale de la signalisation est celle des Smads (Massague & Chen, 2000) ; (Attisano & Wrana, 1998).

Récepteur de type II:

Il existe 5 types de récepteurs de type II appelés (TβRII, ACTRII A et B, BMPRII, AMH-R). Le TGF-β se lie au récepteur TβRII, caractérisé par une région extracellulaire riche en cystéine et d'un domaine intracellulaire dans lequel est situé le domaine kinase sérine /thréonine (Wrana et al, 1994). L'affinité entre le TGF-β et le récepteur TβRII est

extrêmement forte, ainsi, une faible quantité de TGF-β actif est suffisante pour que le complexe se forme (Oh et al, 2000).

Récepteur de type I:

Sept récepteurs de type I de la famille du TGF-β ont été décrits chez les mammifères. Ils sont appelés ALK-1 à ALK-7 (Activin like receptor). Parmi ces récepteurs, la signalisation TGF-β est médiée par les récepteurs TβRI (ALK5) dans la plupart des cellules, ALK-1 dans les cellules endothéliales et ALK2 dans les cellules liées au développement cardiovasculaire (Fig.9) (Goumans et al, 2003). Les récepteurs de type I possèdent un domaine intracellulaire dans lequel est situé le domaine kinase du récepteur. Ce dernier est constitué d'une boucle L45 qui détermine la spécificité des R-Smads aux récepteurs (Derynck & Feng, 1997).

Récepteurs accessoires :

Les récepteurs de type III peuvent soit améliorer l'affinité du TGF-β pour ses autres récepteurs soit au contraire limiter leurs activités. En effet, on compte parmi ces co-récepteurs le betaglycane et l'endogline. La fonction principale de ces récepteurs accessoires est de stabiliser la liaison du TGF-β au TβRII. Cependant, ils peuvent aussi contrôler négativement la signalisation TGF-β. En effet, le betaglycane est la cible d'un clivage protéique associé à l'apparition d'une forme soluble qui aura un rôle antagoniste par rapport à la forme membranaire. Cette forme soluble fixe et séquestre le TGF-β dans le milieu extracellulaire empêchant sa fixation aux récepteurs TβRII (Velasco-Loyden et al, 2004). Le récepteur endogline est principalement exprimé dans les cellules endothéliales et stabilise la liaison des isoformes TGF-β1 et TGF-β3 au TβRII (Cheifetz et al, 1992).

Figure 10 : Tableau des ligands et récepteurs de la signalisation TGF-β (Wakefield & Hill, 2013)

Ligand	Type I receptor	Type II receptor	Co-receptors
Inhibin-α	No type I receptor	ACTRII	ND
Activin-βA	ALK4	ACTRII and ACTRIIB	ND
Activin-βB	ALK4 and ALK7	ACTRII and ACTRIIB	ND
Activin-βE	Unknown receptor	Unknown receptor	ND
Activin-βC	Unknown receptor	Unknown receptor	ND
GDF1	ALK4 and ALK7	ACTRII and ACTRIIB	CRIPTO and cryptic
GDF3	ALK4 and ALK7	ACTRII and ACTRIIB	CRIPTO and cryptic
NODAL	ALK4 and ALK7	ACTRII and ACTRIIB	CRIPTO and cryptic
BMP3	No type I receptor	ACTRIIB	ND
BMP3B (also known as GDF10)	ALK4	ACTRII	ND
GDF11	ALK4 and ALK5	ACTRII and ACTRIIB	ND
Myostatin (also known as GDF8)	ALK4 and ALK5	ACTRIIB	ND
GDF9	ALK4	BMPR2	ND
TGFβ1	ALK1‡ and ALK5	TGFBR2	β-glycan and endoglin
TGFβ2	ALK1 and ALK5	TGFBR2	β-glycan and endoglin
TGFβ3	ALK1 and ALK5	TGFBR2	β-glycan and endoglin
GDF15	Unknown	TGFBR2	ND
BMP9	ALK1	ACTRII and BMPR2	ND
BMP10	ALK1	ACTRII and BMPR2	ND
BMP2	ALK3 and ALK6	ACTRII, ACTRIIB and BMPR2	ND
BMP4	ALK3 and ALK6	ACTRII, ACTRIIB and BMPR2	ND
GDF5 (also known as BMP14)	ALK3 and ALK6	ACTRII, ACTRIIB and BMPR2	ND
GDF6	ALK3 and ALK6	ACTRII, ACTRIIB and BMPR2	ND
GDF7	ALK3 and ALK6	ACTRII, ACTRIIB and BMPR2	ND
BMP5	ALK2, ALK3 and ALK6	ACTRII, ACTRIIB and BMPR2	ND
BMP6	ALK2, ALK3 and ALK6	ACTRII, ACTRIIB and BMPR2	ND
BMP7	ALK2, ALK3 and ALK6	ACTRII, ACTRIIB and BMPR2	ND
BMP8	ALK2, ALK3 and ALK6	ACTRII, ACTRIIB and BMPR2	ND
BMP15	ALK6	BMPR2	ND
AMH	ALK2 and ALK3	AMHR2	ND

Les ligands sont répertoriés selon le type du récepteur utilisé. (ALK1‡) est spécifique de l'endothélium.

2-3-2. Description de la voie de signalisation canonique de TGF-β

Le TGF-β se fixe au récepteur TβRII, forme un complexe hétérodimérique qui recrute le TβRI. TβRII phosphoryle les résidus serines et thréonines de TβRI dans son domaine GS (Shi & Massague, 2003). Le complexe hetero-tétramérique transduit le signal à l'intérieur de la cellule (Feng & Derynck, 2005).

Il existe 8 protéines Smads mais seules 3 fonctions les différencient (Flanders et al, 2001); (Luukko et al, 2001) ; (Attisano & Wrana, 2000). En effet, les Smads régulatrices (R-Smads 1, 2, 3, 5 et 8) interagissent de façon spécifique directement avec le récepteur TβRI activé et sont spécifiques d'un ligand. Le co-Smad (Smad 4) est un médiateur commun pour pour toutes les Smads régulatrices. Les Smads inhibitrices (I-Smad 6, 7) inhibent les R-

Smads (Miyazono et al, 2000). Les R-Smads 2 et 3 sont activées par le récepteur TβRI et transduisent les réponses biologiques de TGF-β ou de l'activine (Massague et al, 2000). Elles présentent plus de 90% d'homologie de séquence en aa. Elles sont composées de deux domaines hautement conservés aux extrémités N et C -terminales (MH1 et MH2). Ces domaines sont reliés par un domaine linker riche en proline de longueur variable (Massague, 2000). Le domaine MH1 est uniquement présent au sein des structures des R-Smad et Co-Smad. Ce domaine est constitué de 130 aa possédant une structure en épingle à cheveu bien conservée entre les différents R et Co –Smad. Via le domaine MH1, Smad3 est capable d'interagir avec l'ADN contrairement à Smad2 qui présente une insertion de 30 aa dans ce domaine empêchant la liaison à l'ADN (Shi et al, 1998). La présence d'un signal de localisation nucléaire (NLS) dans le domaine MH1 joue un rôle dans la localisation nucléaire des protéines R-Smads et Co-Smad. Le domaine MH1 est également impliqué dans l'interaction des Smads avec plusieurs facteurs de transcriptions (Reguly & Wrana, 2003). Le domaine MH2 est constitué d'environ 200 aa, il est impliqué à la fois dans les interactions avec le récepteur TβRI et différentes protéines. Les R-Smads sont activées par phosphorylation du motif SSXS par le récepteur TβRI localisé dans la boucle L3 du domaine MH2 (Souchelnytskyi et al, 1997) ; (Abdollah et al, 1997). Cette boucle L3 est commune aux protéines Smad2, Smad3 et des protéines Smad1, Smad5, Smad8, mais diffère par deux résidus entre les deux groupes. Cette différence explique la spécificité d'interaction des protéines R-Smads avec le TbRI ou Alk1 (Chen et al, 1998) ; (Lo et al, 1998). Les protéines Co-Smad (SMAD4) et I-Smad (Smad6 et Smad7) ne peuvent être phosphorylées car le motif SSXS est absent du domaine C-terminal. Cependant, les I-SMADs sont capables de s'associer fortement aux récepteurs et empêchent la transduction du signal par la voie des R-Smads (Fig.10) (Hayashi et al, 1997) ; (Imamura et al, 1997) ; (Nakao et al, 1997a) ; (Nakao et al, 1997b).

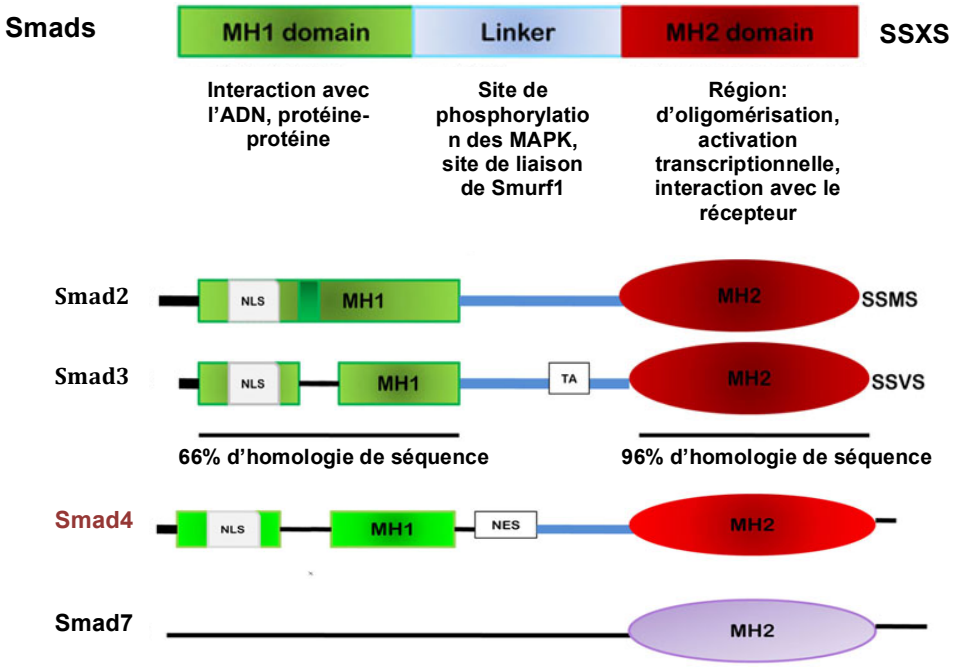

Figure 11: Structures des différentes protéines Smads (Samanta & Datta, 2012)

Les protéines Smads sont composées des domaines MH1 et MH2 liés par une région charnière (linker). Le domaine MH1 de la protéine Smad2 présente 30 aa supplémentaires (indiqués par une zone vert foncé). Smad3 contient un domaine de trans activation (TA) dans la région charnière. Smad4 possède un signal nucleus export (NES) dans la région charnière. Smad2, 3 and Smad4 possèdent une séquence signal de localisation nucléaire (NLS) dans le domaine MH1. Smad 7 ne présente que le domaine MH1. Schéma adapté (Brown et al, 2007)

Les Smad 2/3 phosphorylées, s'associent à l'unique co-Smad, Smad 4. Une fois le complexe formé, celui est transloqué dans le noyau pour former un facteur de transcription qui régule les gènes dépendants des Smads (Fig.11).

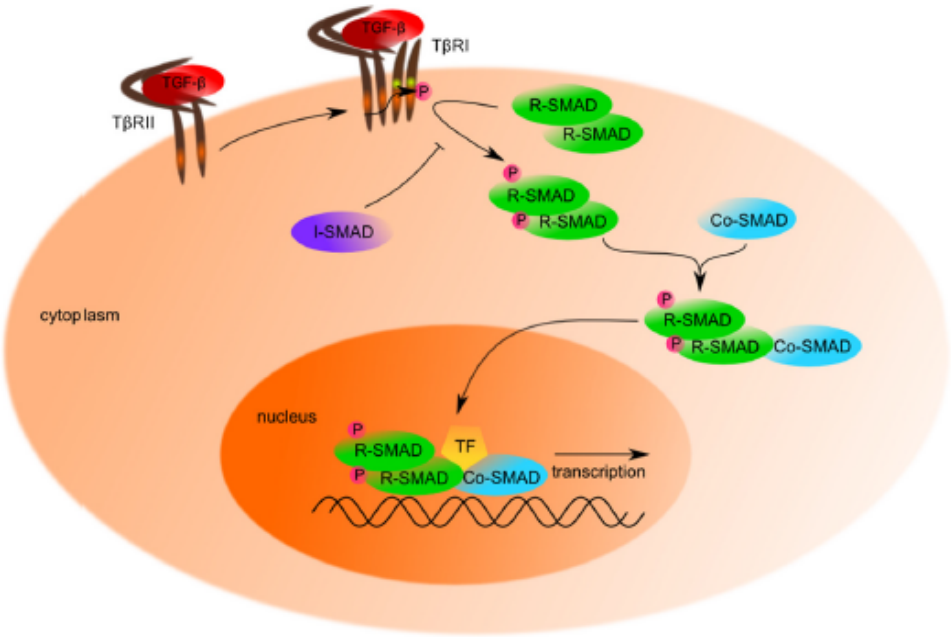

Figure 12 : voie canonique du TGF-β (Kubiczkova et al, 2012)

La fixation du TGF-β au récepteur TGFβ-RII induit l'activation par phosphorylation du TGFβ-RI. Puis le TGF-β-RI phosphoryle les facteurs de transcription de la famille, les R-Smad. Cette phosphorylation permet la formation des complexes R-Smad/coSmad. Les complexes R-Smad/coSmad sont transloqués dans le noyau, où ils se fixent sur les séquences promotrices des gènes régulés par le TGF-β.

2-3-3. La voie non canonique du TGF-β

Les réponses biologiques au TGF-β sont assurées majoritairement par la signalisation des R-Smads mais pas seulement. Les récepteurs au TGF-β sont capables d'induire d'autres voies de signalisations responsables des effets multiples du TGF-β. Ils activent les cascades de signalisation de la voie MAPK (Mitogen –activated protein kinases) telles que ERK1/ERK2, JNK, P38), qui régulent la prolifération, la migration et la différentiation cellulaire (Davies et al, 2005; Zhang, 2009) ; (Xie et al, 2004); (Bakin et al, 2002). Ils activent également la voie PI3K-AKT et la famille des GTPases, en particulier les RhoA, et perturbent l'assemblage des complexes d'adhésion focale et l'organisation des filaments d'actine (Clements et al, 2005) ; (Maddala et al, 2003) ; (Bhowmick et al, 2001; Edlund et al, 2002). Le récepteur TβRII est capable de phosphoryler la protéine Par6, protéine cruciale dans la mise en place du pôle baso-apical des cellules épithéliales, responsable de la dégradation de Rho A et de l'assemblage des jonctions serrées dans la cellule (Fig12) (Moustakas & Heldin, 2009) ; (Moustakas & Heldin, 2007; Ozdamar et al, 2005). Les différentes réponses biologiques au TGF-β sont médiées par plusieurs voies de signalisation ayant en commun le même récepteur.

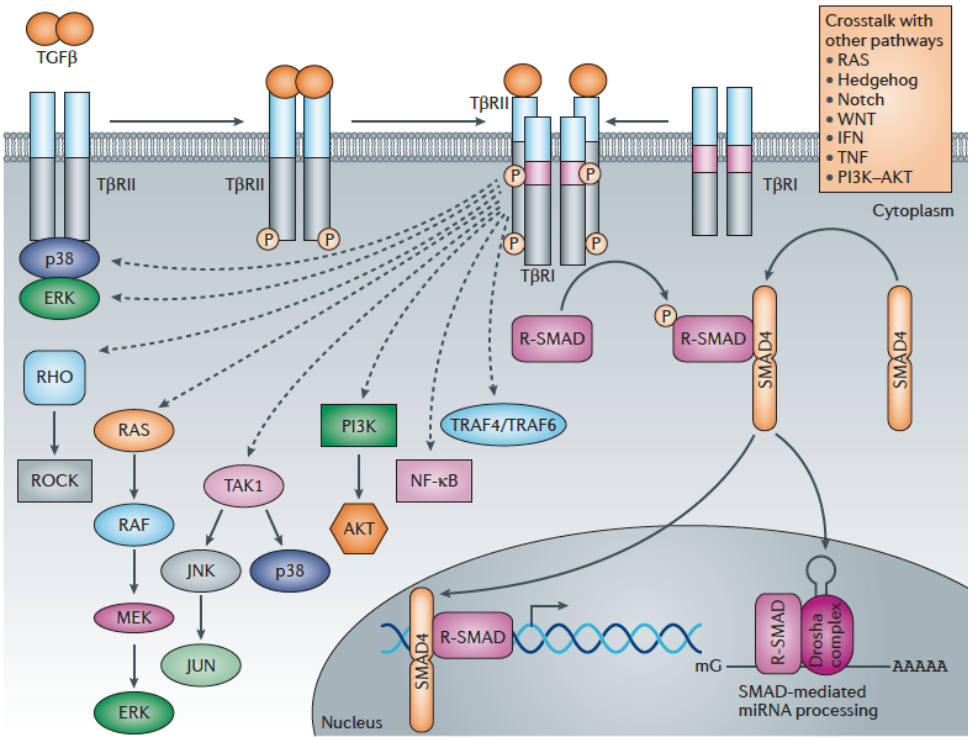

Figure 13 : Voies non canoniques du TGF-β (Akhurst & Hata, 2012)

Une fois le ligand fixé sur son récepteur, d'autres voies de signalisation peuvent être activées telles que RHO, PI3K-AKT, ERK, JNK. La voie de signalisation TGF-β peut être influencée par d'autres voies comme celles de WNT, Hedgehog, Notch, l'interféron, TNF et RAS. L'interaction entre la voie de signalisation de TGF-β avec les autres définit les activités du TGF-β pour propager des signaux spatio-temporels et spécifiques.

2.4. Régulation des voies de signalisation du TGF-β

La complexité des réponses biologiques du TGF-β nécessite que la voie de signalisation soit hautement régulée par différents acteurs. Elle est soumise à différents mécanismes de régulation qui ont lieu au niveau de la membrane, du cytosol et du noyau. En effet, des mécanismes comme l'endocytose, la déphosphorylation, l'ubiquitination des différents acteurs de la voie de signalisation orientent l'expression des gènes cibles et donc les réponses biologiques du TGF-β.

2-4-1. Régulation de la voie de signalisation dans le cytosol

2-4-1-1. Endocytose du complexe de récepteur

Des protéines adaptatrices augmentant l'affinité des Smads pour le complexe ligand – récepteur ont été décrites, en particulier la protéine SARA (Smad anchor for receptor activation), protéine associée aux endosomes précoces. La protéine SARA interagit avec les protéines Smad 2/3 non phosphorylées afin de localiser ces Smads vers la membrane et

favoriser l'activation des Smads par le récepteur TβRI. Pour ce faire, la protéine SARA doit être liée à une protéine cytoplasmique cPML. La présence de cPML est cruciale pour la transmission du signal, car des cellules primaires issues de souris déficientes pour cPML deviennent résistantes aux effets biologiques du TGF-β en terme d'arrêt de croissance et d'apoptose. En effet, l'absence de cPML entraîne un arrêt de la transmission de signal car l'interaction de la protéine SARA avec les protéines Smad 2/3 ne peut se faire (Lin et al, 2004). Par ailleurs, cPML est régulée par une protéine TGIF qui inhibe l'interaction de cPML à la protéine SARA, en séquestrant cPML dans le noyau ce qui conduit à l'inhibition de l'activation des R-Smad (Seo et al, 2006). Une étude récente a mis en évidence que le TGIF est ubiquitinylé par une ubiquitine ligase PHFRI, inhibant ainsi l'interaction de TGIF avec cPML (Ettahar et al, 2013).

La protéine Smad7, régule la signalisation du TGF-β à la fois dans le cytoplasme et dans le noyau. L'action inhibitrice de Smad7 fonctionne comme une boucle de rétrocontrôle négatif, car l'expression de Smad7 est induite par le TGF-β de façon dépendante des Smads (Massague & Chen, 2000). Smad7 est capable de s'associer au récepteur TβRI de manière stable soit pour inhiber la phosphorylation des R-Smad par le récepteur, soit pour dégrader le TβRI en recrutant à la membrane Smurf2, une ubiquitine ligase (Miyazono et al, 2000) ; (Yan et al, 2009). De plus, la liaison de Smad 7 au TβRI permet aussi le recrutement de GADD34, une phosphatase qui conduit à la déphosphorylation du TβRI (Shi et al, 2004). Par ailleurs, d'autres cytokines comme l'EGF, l'IFN ou le TNF augmentent l'expression de Smad 7 et donc diminuent les réponses au TGF-β.

L'endocytose du complexe TβR est un mécanisme hautement régulé. En effet, le récepteur est internalisé principalement selon deux voies : (1) la voie d'endocytose clathrine dépendante permettant l'activation de Smad2 et Smad3 et un recyclage du récepteur, (2) la voie d'endocytose dépendant des cavéolines, associée à la présence de Smad7 et Smurf2 qui conduit à un arrêt de la signalisation associée aux protéines R-Smads et provoque la dégradation du récepteur (Le Roy & Wrana, 2005).

2-4-2. Régulation au niveau de la translocation nucléaire

2-4-2-1. Impact de la phosphorylation des Smads sur leur localisation subcellulaire

La phosphorylation des R-Smad dans le domaine MH2 par le récepteur TβRI n'est pas la seule région qui régule l'activité des R-Smad. D'autres protéines kinases ERK, JNK, p38 et CDK ont également la capacité de phosphoryler les R-Smads dans leur région charnière (Heldin & Moustakas, 2012). Ces phosphorylations inhibent la translocation de Smad3 phosphorylé dans le noyau. En conséquence, il a été montré que ces phosphorylations inhibaient les transcriptions dépendantes de Smad3 et l'arrêt du cycle cellulaire induit par le TGF-β (Kamato et al, 2013) ; (Millet et al, 2009) ;(Feng & Derynck, 2005). Il a également été montré que la phosphorylation des Smad 2/3 dans la région charnière par JNK était associée à l'augmentation de la fonction pro-tumorale du TGF-β dans le cancer colorectal (Yamagata et al, 2005). De plus la protéine kinase Akt interagit aussi avec Smad3 non phosphorylé et influence sa liaison au TβRI (Feng and Derynck, 2005). Mais AKT est aussi capable de phosphoryler Smad2/3 dans sa région charnière et bloquer la translocation nucléaire du complexe Smad3/ Smad4 (Guo and Wang, 2009 ; (Zhang, 2009); Remy et al, 2004). Plusieurs autres kinases activées par des facteurs de croissances tels que HGF ou EGF conduisent à l'activation de la protéine Ras et à la phosphorylation des protéines R-Smads par les MAPK (Derynck & Zhang, 2003) ; (Yoshida et al, 2005)}. L'activation des voies de signalisation impliquées dans la survie cellulaire entraîne une diminution de la translocation des R-Smad dans le noyau, atténuant ainsi les transcriptions dépendantes de Smads.

L'exportation nucléaire des R-Smads est régulée par leur état de déphosphorylation et la dissociation du complexe R-Smad/Smad 4 (Fig.13) (Shi & Massague, 2003) (Inman et al, 2002) ;(Xu et al, 2002) ; (Izzi & Attisano, 2004). Ainsi, la phosphatase PPM1A/PP2Cα déphosphoryle les R-Smads dans le noyau et facilite l'exportation nucléaire des R-Smads (Lin et al, 2006) ; (Hill, 2006). Cette interaction peut être stabilisée par PTEN et faciliterait donc la déphosphorylation des R-Smads (Bu et al, 2008). Une autre protéine, MTMR4, attenue également la signalisation TGF-β en réduisant le niveau de phosphorylation des R-Smads dans les endosomes précoces (Yu et al, 2010). Cette protéine module également la signalisation BMP *via* la déphosphorylation de Smad1 (Yu et al, 2013). L'équipe de Bruce et al, a identifié une protéine PP5, une phosphatase capable d'interagir avec Smad2/3 et de les déphosphoryler (Bruce et al, 2012). Cette même équipe remet en cause la fonction phosphatase de PPM1A. PPM1A a été décrite capable de déphosphoryler Smad2/3 dans le noyau alors que l'équipe Bruce et al, la retrouve localisée dans le cytoplasme de plusieurs

lignées (Bruce & Sapkota, 2012). La régulation de la signalisation TGF-β par les phosphatases reste un sujet controversé dont il est nécessaire d'éclaircir les mécanismes.

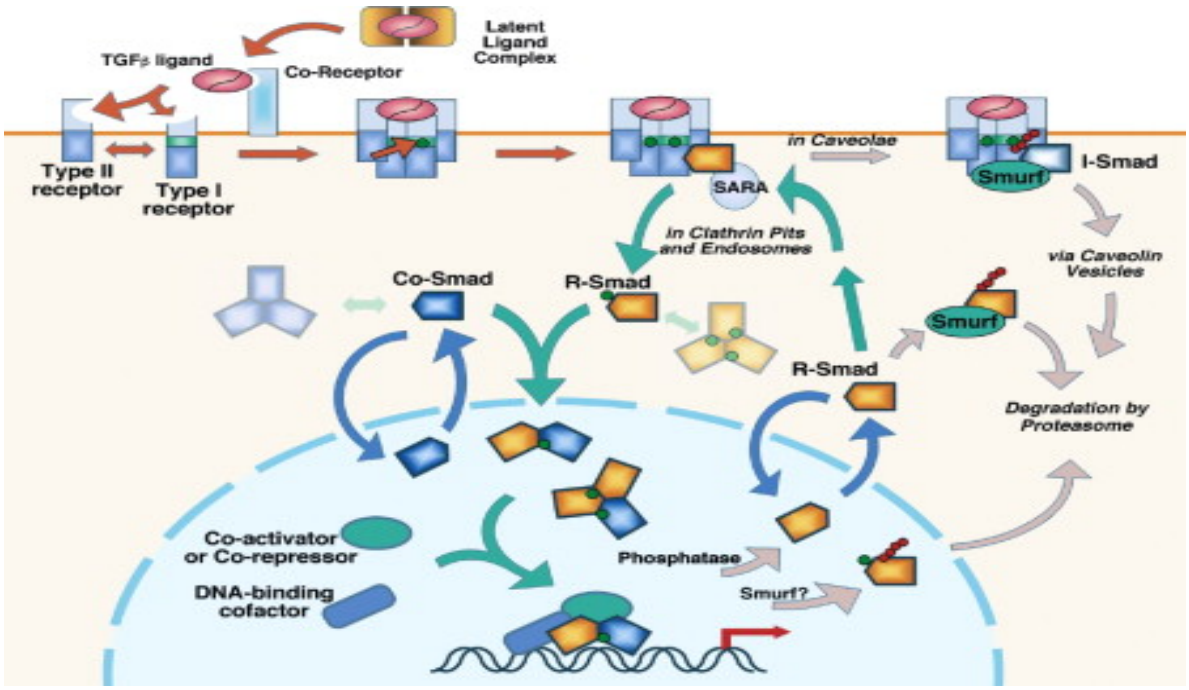

Figure 14 : Schéma de la régulation cellulaire de la voie de signalisation TGF-β (Shi & Massague, 2003).

Schéma de la régulation de la voie de signalisation TGF-β de la membrane cellulaire au noyau. Les flèches indiquent les flux le code couleur est rouge pour ceux du ligand et le récepteur activé, le gris pour celui des Smads et du récepteur non activé, le vert pour celui des Smad activées et la formation de complexes, le bleu indique le trafic nucléo –cytoplasmique des Smads. Les petits cercles rouges désignent l'ubiquitination et ceux en vert la phosphorylation.

2-4-2-2. Régulation par ubiquitination et ubiquitination-like

L'ubiquitination est un processus qui régule, le trafic, la localisation, de certaines protéines et les voies de signalisation cellulaire dont celles du TGF-β. L'ubiquitination est une modification post-traductionnelle de protéines au cours de laquelle une ou plusieurs protéines d'ubiquitine se lient par des liaisons covalentes à une protéine cible, sur une lysine pour être dégradée dans le protéasome. Le devenir de la protéine est fonction de son degré d'ubiquitination qui l'oriente vers une protéolyse cellulaire (Mukhopadhyay & Riezman, 2007). L'ubiquitination fait intervenir successivement trois enzymes appelées ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) et ubiquitine ligase (E3). La spécificité de l'ubiquitination dépend majoritairement de l'E3 ligase qui détermine la protéine cible. La protéine ubiquitine-ligase Arkadia est à la fois un régulateur positif et négatif de la voie de signalisation TGF-β. En effet, cette protéine ubiquitine les Smad2/3 phosphorylées et

conduit à l'arrêt du signal (Mavrakis et al., 2007). D'un autre coté la protéine Arkadia a été identifiée pour induire la dégradation de Smad7 et SnoN, favorisant ainsi la voie de signalisation Smad dépendante (Levy et al., 2007; Nagano et al., 2007) (De Boeck & ten Dijke, 2012). Les protéines Smurf 1 et 2 sont des E3 ubiquitine ligases qui régulent négativement la signalisation TGF-β. La protéine Smurf1 régule spécifiquement les effecteurs Smad1 et 5 impliquées dans la voie de signalisation des BMP (Murakami et al, 2003; Zhao et al, 2004; Zhu et al, 1999). La protéine Smurf2 interagit avec les protéines les R-Smads *via* le domaines PY des protéines Smads (Kavsak et al, 2000; Lin et al, 2000). En effet, Smurf2 mono-ubiquitine uniquement la forme phosphorylée de smad3 en tyrosine179 empêchant ainsi la formation du complexe Smad 3/Smad4, et induit aussi la dégradation de Smad2 (Lin et al, 2000)Tang and Zhang, 2011; Tang et al., 2011). De plus, Smuf2 influence la localisation de Smad7 dans le noyau, dégrade le récepteur TβRI et module l'endocytose du complexe des récepteurs. L'effet inhibiteur de Smad7 est renforcé par la stabilisation de son interaction avec TβRI par les protéines STRAP (*Serine-Threonine kinase Receptor-Associated Protein*) ou YAP-65 (*Yes-Associated Protein* 65) (Datta et al, 1998) ; (Ferrigno et al, 2002). La protéine AIP4/Itch, E3 ubiquitin ligase (Athophin Interacting Protein 4) se lie spécifiquement à Smad7 et favorise son association avec le récepteur TβRI activé et inhibe ainsi la signalisation TGF-β (Lallemand et al, 2005). Des cytokines comme l'IFN-γ (Interféron gamma) ou le TNF-α (Tumor Necrosis Factor alpha) régulent l'expression de Smad7 et participent à l'atténuation de la réponse au TGF-β (Ulloa et al, 1999) ; (Kuga et al, 2003). Une autre E3 ligase est impliquée dans la voie du TGF-β, la protéine TRIM33 (Tripartite-containing Motif 33) ou TIFγ (Transcriptional Intermediary Fractor 1γ). Cette protéine agit comme un régulateur positif (He et al, 2006) ou négatif (Dupont et al, 2005) de la voie de signalisation TGF-β. En effet la protéine Smad4 peut être la cible de l'ubiquitine ligase TIF1γ qui bloque la formation du complexe Smad4/R-Smad et inhibe donc la voie de signalisation du TGF-β (Dupont et al, 2005) (Morsut et al, 2010) ; (Agricola et al, 2011). De plus, TIF1γ pourrait aussi interagir fortement avec les protéines Smad2/3 phosphorylées et ainsi réguler les effets biologiques du TGF-β (He et al, 2006).

L'ubiquitination est un mécanisme réversible médié par des protéines dé-ubiquitine ligases comme UCH37 et USP15 (DUB) (Fig.15) (Dupont et al, 2009).

Une autre modification post traductionnelle, la sumoylation peut réguler la voie du TGF-β. Ce mécanisme aboutit à la liaison covalente d'une ou plusieurs protéines SUMO sur une lysine acceptrice d'une protéine cible. Contrairement à l'ubiquitination, la sumoylation n'est

pas un système où les protéines sont dégradées. La protéine Smad4 peut être sumoylée à deux endroits différents assurant la stabilité et les transcriptions dépendantes des Smads (Lin et al, 2003a); (Lin et al, 2003b) ; (Lee et al, 2003). De plus, le récepteur TβR1 peut aussi être la cible d'une sumoylation provoquant le recrutement, l'activation de Smad3, et favorise ainsi les réponses pro-tumorales du TGF-β (Kang et al, 2008).

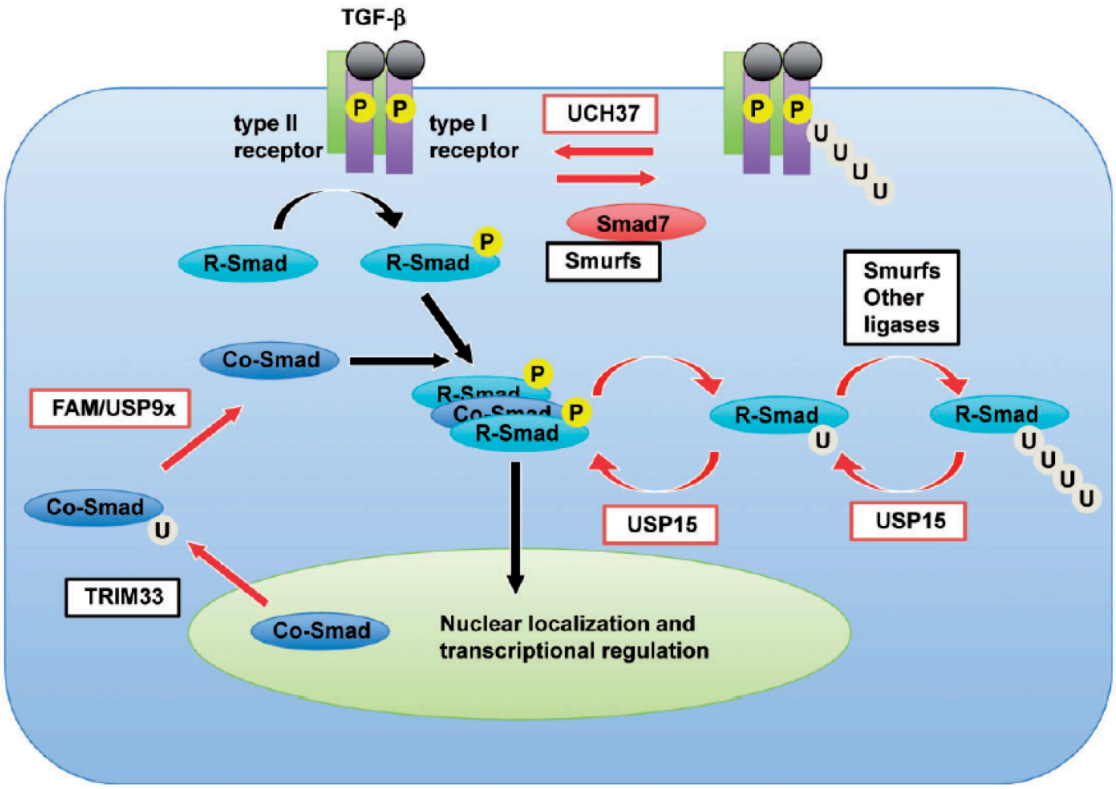

Figure 15 : Régulation de la signalisation TGF-β par ubiquitination et dé-ubiquitination.

Le récepteur TβRI et les R-Smads sont ubiquitinylés par les protéines SMURFs. UCH37 et USP15 sont deux protéines responsables de la dé-ubiquitination du TβRI et les R-Smads.

2-4-3. Régulation au niveau nucléaire :

L'activation transcriptionnelle spécifique des gènes cibles des Smads est finement régulée et requiert le recrutement de facteurs supplémentaires comme des facteurs de transcriptions ou des co-activateurs tel que CBP (*CREB*-binding Protein) et p300. En effet, pour initier la transcription, Smad3 se lie à CBP ou p300, des histones acétyltransférases qui vont favoriser une décondensation de la chromatine. Cette liaison est dépendante d'une phosphorylation de CBP/p300 par MSK1, elle-même activée par p38 (Abecassis et al, 2004). A l'inverse, des répresseurs transcriptionnels qui possèdent une activité histone désacétylase permettent à la chromatine de revenir à un état plus condensé. Parmi ces corépresseurs, SKI et Sno ou TGIF sont connus comme régulateurs négatifs de la voie de signalisation de TGF-

β (Seo et al, 2004). En fonction du type cellulaire, l'équilibre entre les Co-activateurs et les corépresseurs module la transcription génique (Fig.16).

La protéine Smad3 interagit avec une séquence ADN 5'-CAGAC-3', appelé SBE (Smad Binding Element) mais si cette fixation est nécessaire, elle n'est pas suffisante pour provoquer une transcription spécifique d'un gène cible. Cette transcription est dépendante de la fixation d'autres facteurs de transcription dans le promoteur de ce gène. En conséquence, l'activation des voies de signalisation autres que les Smads détermine l'activation et la fixation de facteurs de transcription dans un gène cible du TGF-β. Par exemple, l'activation de la voie PI3K/AKT phosphoryle le facteur FOXO3 et empêche sa translocation nucléaire (Brunet et al, 1999). Or la fixation de FOXO3 et de Smad3 sur le gène p21 est nécessaire pour provoquer la transcription de ce gène (Seoane et al, 2004). L'activation d'AKT inhibe donc l'expression de ce gène qui est impliqué dans l'arrêt de croissance de ces cellules. Plus généralement, pour un gène cible particulier, des partenaires différents comme les facteurs de transcription AP1 ATF3, etc., en s'associant aux Smads établissent des interactions de haute affinité et très sélectives avec les séquences promotrices de ce gène. Les réponses au TGF-β sont donc liées à des interactions fonctionnelles avec de multiples voies de signalisation.

Cette régulation explique les effets différents du TGF-β puisque l'activité transcriptionnelle des gènes cibles du TGF-β sera dépendante du type cellulaire (présence ou non d'un facteur de transcription, de Co-activateurs ou répresseurs) ainsi que l'état de la cellule (activation de différentes voies de signalisation)

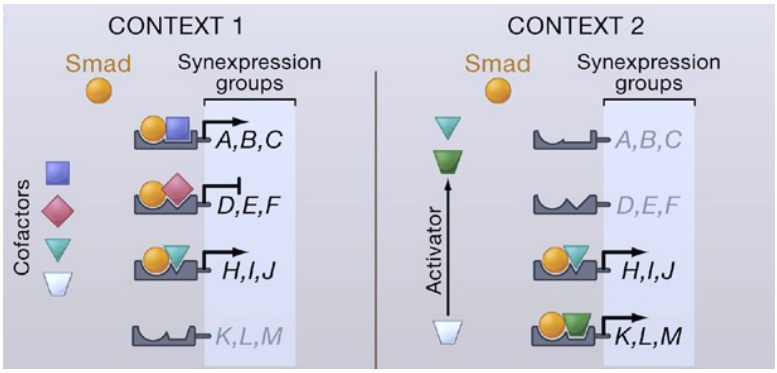

Figure 16: Représentation des différentes combinaisons des facteurs de transcription

Suivant le contexte cellulaire (type cellulaire ou condition), la combinaison des différents R-Smads avec des co facteurs activateurs détermine la transcription d'un set de gènes (Massague, 2008).

Chapitre III :

Le TGF-β dans les pathologies du foie

3. Le TGF-β

Actif sur tous les types cellulaires, le TGF-β contrôle la prolifération, la différenciation, la motilité, l'adhésion, l'apoptose, selon le type cellulaire et l'état de différenciation. Ces fonctions participent au contrôle du développement et de l'homéostasie des tissus. Des perturbations de la voie de signalisation sont impliquées dans diverses pathologies comme la fibrose et le cancer.

3-1. Les effets du TGF-β sur l'arrêt de croissance et l'apoptose

L'un des premiers effets du TGF-β est l'arrêt du cycle cellulaire en phase G1 et une inhibition de croissance de nombreuses cellules. La voie de signalisation des Smads est capitale dans la transduction du signal de l'arrêt du cycle cellulaire. L'inhibition de la croissance cellulaire implique différents mécanismes comme l'induction des CDK inhibiteurs, p21, $p27^{KIP1}$ (Polyak et al, 1994), $p15^{INK4B}$ (Hannon & Beach, 1994) ou encore l'inhibition de l'expression de c-myc et la déphosphorylation des protéines Cdc 25 (Fig.17) (Isoe et al, 1998; Kang et al, 2003). Le TGF-β réprime aussi l'expression des protéines Id (*Inhibitor of Differentiation Inhibitor of DNA-binding 1-gene*), facteurs nucléaires qui contrôlent négativement la sortie du cycle cellulaire et l'entrée en différenciation de divers types cellulaires (Kang et al, 2003) ; (Siegel & Massague, 2003). Au cours de la régénération hépatique, plusieurs facteurs de croissance tels que EGF, HGF favorisent la prolifération des hépatocytes. Quelques heures après le processus, une hausse de l'expression du TGF-β est observée. Le TGF-β joue un rôle important dans ce processus en inhibant la prolifération des hépatocytes à la fin de la régénération hépatique (Karkampouna et al, 2012).

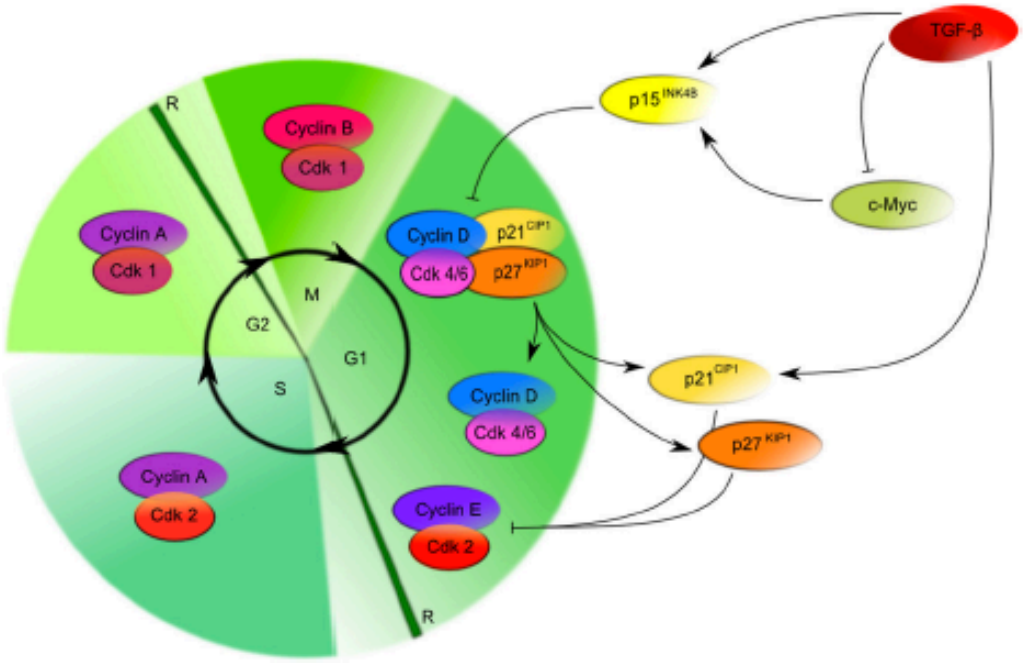

Figure 17 : Régulation du cycle cellulaire par le TGF-β

Le TGF-β induit l'expression de p15 ^{INK4B} et réprime l'expression de c-Myc qui inhibe la formation du complexe cyclinD- CDK4/6. De plus une faible expression de c-Myc induit l'expression de p15 ^{INK4B} et p21 ^{CP1}. La libération de p21^{CPI} et p27^{KP1} inhibe le complexe CyclineE /Cdk2 responsable du passe G1/S.

3-2. Les effets pro-tumoraux du TGF-β

Au cours du développement tumoral, les cellules malignes modifient leurs réponses biologiques au TGF-β. En effet, les cellules deviennent résistantes aux effets cytostatiques du TGF-β mais restent sensibles aux effets pro-tumoraux. Cette résistance est expliquée par la présence de mutations génétiques des composants de la signalisation TGF-β mais aussi par l'activation accrue dans les cellules tumorales des voies de signalisation qui inhibent les Smads (fig.18) (Biswas et al, 2004) ; (Wakefield & Roberts, 2002). Le TGF-β facilite la prolifération des cellules tumorales et la dissémination en stimulant, des cytokines pro-angiogéniques comme le CTGF, VEGF et l'ANGPTL4 (Heldin et al, 2012; Padua et al, 2008), le remodelage du microenvironnement (MMP et les TIMP) (Padua and Massagué, 2009) et des facteurs de transcription comme KLF8 (Zhang et al, 2013).

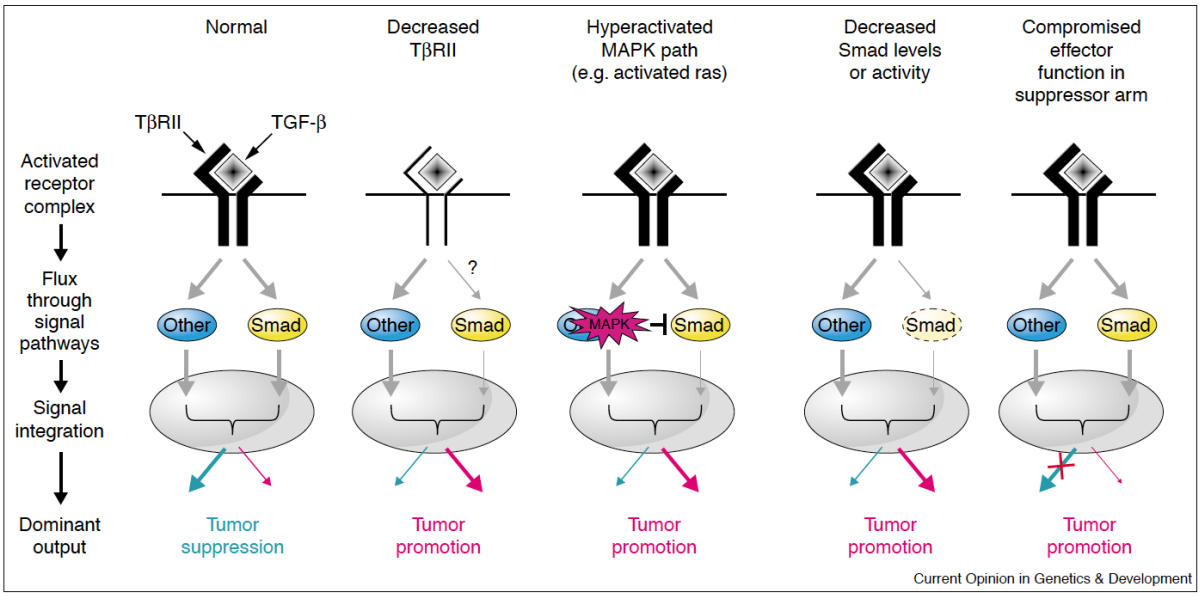

Figure 18: Atténuations de la voie des Smads et promotion de tumeurs

La mutation du TβRII, ou une activation de la voie des MAPK conduit à une diminution de la voie des Smads conduisant à l'effet pro-tumoral du TGF-β (Wakefield & Roberts, 2002).

3-3.La TEM dans le foie

Plusieurs types cellulaires peuvent subir une TEM partielle ou totale. Plusieurs études témoignent qu'un traitement au TGF-β induit une diminution des marqueurs épithéliaux, comme la E-Cadhérine et une augmentation des marqueurs mésenchymateux, tels que Snail1, Smooth Muscle Actin (SMA) (Gonzalez & Medici, 2014). Des cellules du foie comme les cholangiocytes, peuvent subir en culture une TEM (Sato et al, 2007); (Liu et al, 2012). En effet, des cholangiocytes immatures co-cultivés avec des myofibroblastes ou traités au TGF-β subissent une TEM (Rygiel et al, 2008);(Harada et al, 2009); (Chu et al, 2011).

3-3-1. Mécanisme d'induction de la TEM par le TGF-β

La TEM est un processus dynamique au cours duquel les cellules épithéliales perdent leurs propriétés épithéliales pour développer des caractéristiques de cellules mésenchymateuses. Les cellules épithéliales sont des cellules polarisées, adhérentes à la MEC et aux cellules voisines *via* des interactions intercellulaires comme les desmosomes et les jonctions serrées. L'ensemble de ces cellules cohésives s'organise en couche pour former un tissu, l'épithélium. La TEM est un enchainement de plusieurs étapes impliquant un changement structural et fonctionnel complexe, 1) diminution de la polarité basolatérale et l'adhésion cellulaire, 2) le développement de cellules « souche- like » et initiation de l'activité tumorale, résistance à

l'apoptose induite, développement de la migration et invasion cellulaire. En effet, la mise en place de ce processus n'a pas lieu de façon instantanée. La TEM partielle, est une étape intermédiaire où les cellules expriment à la fois les marqueurs épithéliaux et les marqueurs mésenchymateux (Fig.19) (Kalluri & Weinberg, 2009). Ce mécanisme de TEM aboutit à la formation de métastases (Morrison et al, 2013; Taylor et al, 2010). Il existe trois types de TEM, la TEM de Type 1 est associée à l'embryogenèse pour la formation des tissus, organes. La deuxième, de Type 2, est essentielle pour le maintien de l'homéostasie des tissus en induisant la cicatrisation et le remodelage tissulaire lors de dommages. La TEM de Type 3 est cruciale pour faciliter la propagation des cellules tumorales (Kaimori et al, 2010 ; Taylor et al, 2010).

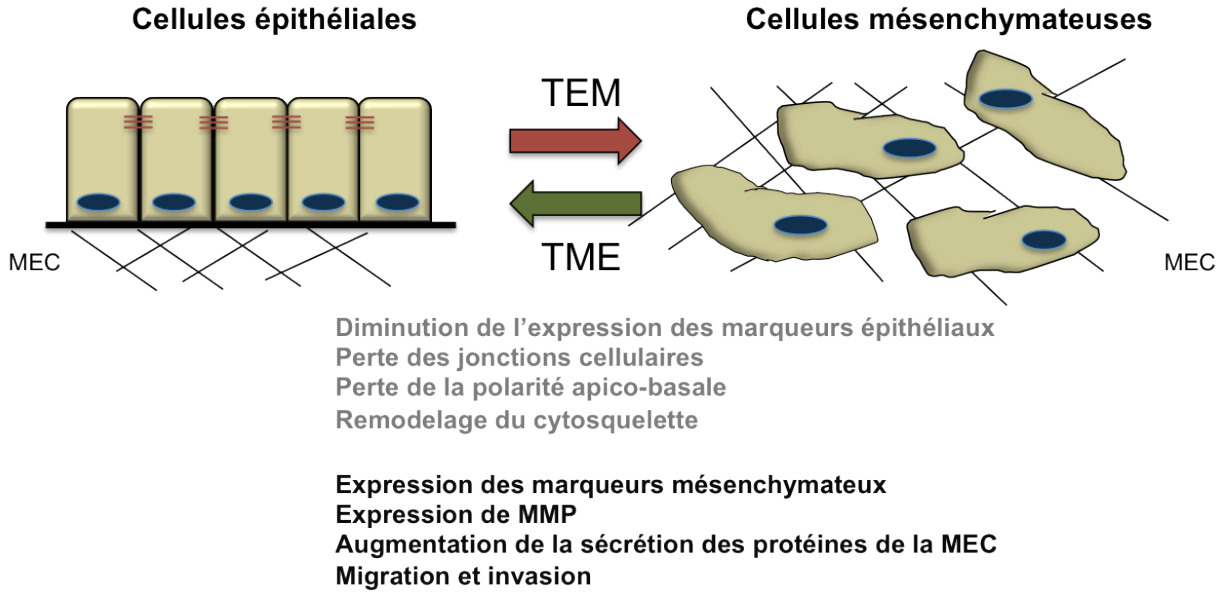

Figure 19 : Schéma de la Transition Epithélium - Mésenchyme.

Phénomène biologique conduisant une cellule épithéliale polarisée à subir des modifications moléculaires la conduisant à perdre son phénotype épithélial et à acquérir celui de cellules mésenchymateuses. Ce processus est réversible, on parle alors de la Transition Mésenchymo-Epithéliale (TME) (Samarasinghe, 2013)

L'initiation de la TEM débute par la régulation de l'expression et de l'activité des facteurs de transcriptions de la TEM. Ces facteurs de transcription régulés par le TGF-β sont Snail, ZEB, Twist, Six 1 (Six family of homeobox), FoxC2 (Forkhead) et les membres de la famille des protéines à grande motilité (HMG2a). Les facteurs de transcription Snail et ZEB répriment la E-cadhérine, molécule essentielle dans l'adhésion cellulaire et induisent l'expression des gènes mésenchymateux. De plus, les membres de la famille Twist induisent principalement l'expression des gènes pro-invasifs et mésenchymateux (Nieto, 2011). La voie de signalisation canonique du TGF-β est essentielle pour réguler la TEM, les protéines R-Smad

induisent l'expression de HMGA2, qui à son tour se lie au R-Smad et facilite ainsi l'expression des facteurs de transcription Snail et Twist (Thuault et al, 2008) (Moustakas & Heldin, 2012) (Morishita et al, 2013). En revanche les gènes épithéliaux régulant l'expression des molécules d'adhésion ou de jonctions serrées sont réprimés par l'interaction du complexe Smad3/4 avec Snail1 (Vincent et al, 2009). Le TGF-β induit également l'activation d'autres voies de signalisation telles que la voie MAP Kinase, la voie PI3K/mTor et d'autres effecteurs comprenant les protéines à tyrosine kinase (Src, Fak) qui jouent aussi un rôle important dans l'induction de la TEM (Xu et al, 2009) ; (Heldin et al, 2012), (Morrison et al, 2013). De plus, la coopération entre les différentes voies non canoniques, les effecteurs du TGF-β et les facteurs de croissance comme l'EGF, le PDGF et l'HGF promeuvent les activités oncogéniques de cette cytokine. La TEM représenterait un processus central dans la dissémination intra-hépatique et la formation de métastases distales (Firrincieli et al, 2010).

3-3-2. Le TGF-β et la fibrose hépatique

La fibrose hépatique est principalement liée à l'activation des cellules étoilées hépatiques (CEH). Les cellules étoilées subissent un processus d'activation. Leur phénotype quiescent, lipocytaire évolue vers un phénotype de type myofibroblastique; les cellules perdent alors leurs vésicules lipidiques, acquièrent un cytosquelette contractile caractérisé en particulier par l'expression de l'alpha-actine muscle lisse(αSMA) et sécrètent la majorité des protéines de la matrice extra-cellulaire (MEC) (Arenson et al, 1988; Friedman, 2008). Le TGF-β est un médiateur majeur de la fibrose. En effet le TGF-β provoque d'une part, l'activation des cellules étoilées en myofibroblastes, et d'autre part, l'expression des protéines de la MEC et l'expression des inhibiteurs des métallo-protéases (TIMP) afin de réduire la dégradation de la MEC (Arthur, 2000 ; Friedman, 2008). L'activation des CEH dépend de la signalisation TGF-β médiée par la voie des Smad3 (Liu, 2003 ; Uemura et al, 2005). Le TGF-β contribue avec d'autres cytokines comme le PDGF (platelet-derived growth factor) à l'activation des CEH. Ces coopérations induisent la phosphorylation des R-Smads dans la région charnière ce qui favorise les réponses biologiques au TGF-β pour le développement de la fibrose (Yoshida & Matsuzaki, 2012).

Plusieurs études *in vitro* et *in vivo*, montrent que les CEH sont la source essentielle de myofibroblastes hépatiques ; d'autres études suggèrent que les hépatocytes joueraient un rôle dans le développement de la fibrose. Ces dernières montrent en effet, que, *in vitro*, les hépatocytes et les cellules épithéliales biliaires subissent une TEM et peuvent participer au

développement de la fibrose par la TEM (Dooley et al, 2008; Meindl-Beinker & Dooley, 2008; Rowe et al, 2011; Rygiel et al, 2008 ; Zeisberg et al, 2007) (Kaimori et al, 2007 ; Nitta et al, 2008). Des études *in vivo* sont en accord avec les observations *in vitro*. Il a été mis en évidence dans un modèle de fibrose murin induit par le CCL4 que les hépatocytes subissaient une TEM (Xue et al, 2013; Zeisberg et al, 2007). Dans un modèle animal de fibrose induit par le CCL4, l'évolution de la fibrose est réduite lorsque que les souris transgéniques surexpriment la protéine Smad7 uniquement dans les hépatocytes (Dooley et al, 2008). De plus, au cours du remodelage tissulaire dans un modèle de fibrose chez la souris, l'expression du facteur transcriptionnel Snail est augmentée. Ce facteur de transcription joue un rôle clé dans la progression de la fibrose hépatique *in vivo* en déclenchant des processus de la TEM (Rowe et al, 2011).

Le concept de la TEM est très discuté *in vivo* : son identification reste difficile puisqu'elle est réalisée par des marquages d'immunohistochimie (IHC) à partir de tissus, ce qui permet seulement d'étudier l'expression d'une protéine à un instant T. Pour contourner ce problème, des techniques de suivi de lignée ont été développées *in vivo*. L'équipe de Zeisberg et al, a développé un modèle de souris double transgéniques traitées au CCL-4 afin d'induire une fibrose. Ce système permet de suivre l'évolution des hépatocytes et met en évidence que ces cellules développent des caractéristiques de cellules mésenchymateuses suggérant une TEM (Zeisberg et al, 2007).

Cependant l'hypothèse que les hépatocytes participent à la TEM au cours de la fibrose est discutée. L'équipe de Taura a mené des études *in vivo* chez des souris transgéniques Contrairement aux résultats publiés, elle met en évidence que les cholangiocytes ne subissent pas une TEM. Plusieurs autres études concluent également que les hépatocytes ne subissent pas une TEM *in vivo* (Chu et al, 2011; Scholten et al, 2010 ; Taura et al, 2010). Le rôle de la TEM dans le développement de la fibrose hépatique est donc encore controversé.

3-3-3. Le TGF-β et l'effet immunosuppresseur

Le TGF-β joue un rôle majeur et crucial dans la régulation de la réponse immunitaire. En effet, des souris déficientes en TGF-β1 développent des pathologies inflammatoires sévères, touchant différents organes responsables de la mort des animaux dès trois semaines de vie (Kulkarni et al, 1993). De plus l'inactivation de la voie de signalisation du TGF-β dans les lymphocytes T induit des symptômes similaires à la privation de la cytokine révélant l'activité régulatrice majeure du TGF-β au niveau des lymphocytes T. Le TGF-β joue un rôle

dans l'ontogénèse des différents sous-types de lymphocytes. Une fois que les lymphocytes T naïfs (Th0, Thelper) reconnaissent l'antigène présenté par les cellules présentatrices d'antigènes, elles peuvent se différencier en Th1, Th2, Th17 et Treg (Nguyen et al, 1995). La différentiation des Th0 est fonction des cytokines présentes dans leur environnement (Fig.19). La différentiation en Th1 oriente le type de réponse immunitaire vers une réponse à médiation cellulaire conduisant à l'activation des macrophages et à la génération des lymphocytes cytotoxiques (CTL). En revanche, les cytokines produites par les lymphocytes Th2 orientent la réponse immunitaire vers une réponse humorale favorisant l'activation des lymphocytes B, conduisant à la production d'anticorps.

Le TGF-β inhibe la prolifération des lymphocytes B et T et altère la différentiation des cellules T (Li et al, 2006), (McKarns et al, 2004). Le TGF-β réprime la différenciation Th1 (Li & Flavell, 2008) et la différenciation Th2 (Gorelik et al, 2000). De plus, le TGF-β régule la différentiation des des lymphocytes en Th17 et Tregs. Les Th17 ont des propriétés pro-inflammatoires sur de nombreux types cellulaires, en particulier, par la production de l'interleukine 17 (IL17). Les Tregs inhibent les réponses des lymphocytes effecteurs ou les font entrer en apoptose, par différents mécanismes. Les lymphocytes pro-inflammatoires Th17 et les lymphocytes régulateurs Tregs ont des fonctions antagonistes et le développement des Th17 s'accompagne de l'inhibition des Tregs, et inversement. L'induction et le maintien de ces deux types de lymphocytes antagonistes sont étroitement régulés: de faibles doses de TGF-β en synergie avec l'IL6 et l'IL21 vont entrainer l'expression de deux facteurs de transcription RORα et Rorγt favorisant ainsi le développement des Th17 (fig.20) (Korn et al, 2009). IL6 ou IL21 en coopération avec le TGF-β participent aussi à la différentiation des Th17 par l'expression d'un autre facteur de transcription STAT3 (Qin et al, 2009). Au contraire des doses plus élevées de TGF-β en synergie avec des cytokines comme IL10 ou IL2 vont entrainer l'expression d'un autre facteur de transcription, FoxP3 responsable de la différentiation des lymphocytes T CD4+ en Treg (Zhou et al, 2008). Les taux de TGF-β sont élevés dans le micro-environnement tumoral ce qui va favoriser la différentiation et le recrutement des Tregs, ce qui pourrait expliquer la tolérance tumorale. L'expression de Foxp3 est maintenue par d'autres facteurs comme IL2, STAT5 (Murawski, 2005), l'acide rétinoic (XU, 20110), et IL6 et IL4 (Yates 2007).

Le foie est aussi le siège de NKT (Natural killer T cell), ce sont des cellules dont le phénotype est intermédiaire entre les NK et les lymphocytes. La contribution dans le développement de la fibrose est positive ou négative. Le TGF-β contrôle la différentiation

des cellules iNKT (invariant Natural killer T cell) qui sont un sous type des cellules NKT. En effet, l'état de signalisation du TGF-β oriente les différentes étapes de la maturation des iNKT. La signalisation médié par Smad4 permet la maturation des iNKT alors que la signalisation Tifγ ou tifγ/Smad permet la prolifération des iNKT (Doisne, 2009)

La TGF-β joue donc un double rôle dans la régulation du système immunitaire, d'un coté le TGF-β contribue à l'immunosuppression en contrôlant la différentiation des lymphocytes Tregs, d'un autre coté, le TGF-β stimule la différenciation des Th17 qui favorisent la réponse pro-inflammatoire (Karimi-Googheri et al, 2014).

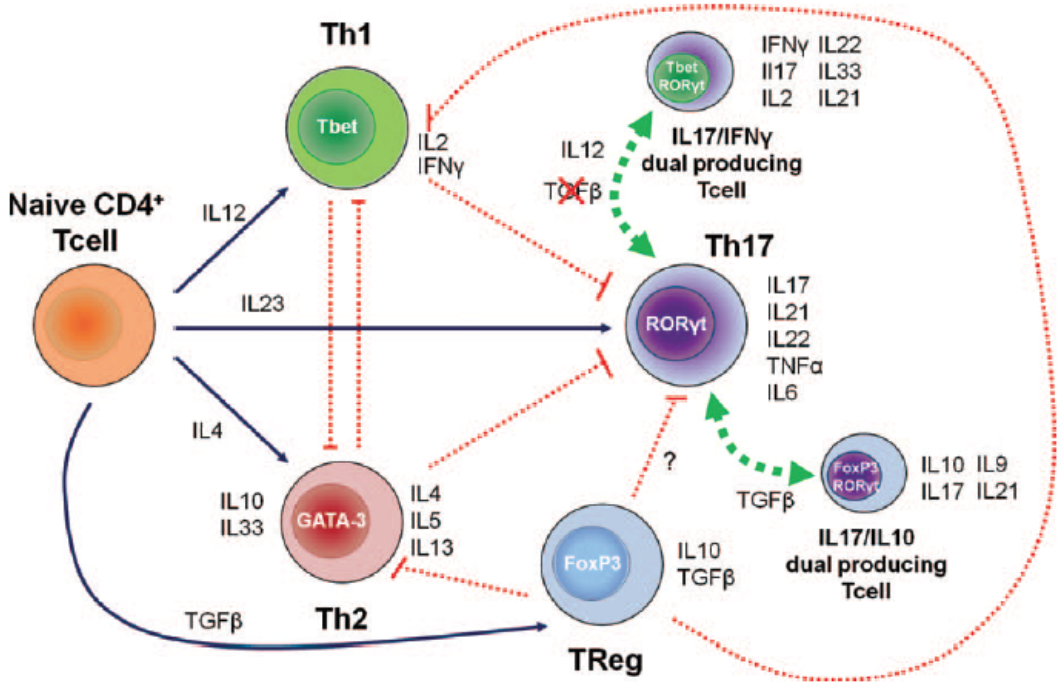

Figure 20 : Régulation des lymphocytes T*helper* (Liuzzo et al, 2013)

Sous l'influence de différents stimuli, les lymphocytes T CD4+ se différencient en T *helper* (Th1, Th2, Th17) ou Treg. Chaque type de lymphocytes est caractérisé par l'expression de facteur de transcription et la sécrétion de cytokines spécifiques. IL-23 induit la différentiation des cellules Th17 produisant de l'IL17. Les cytokines induites par les cellules Th1 ou Th2 inhibent la différentiation des $CD4^+$ en Th17. Le TGF-β induit la différentiation des cellules $CD4^+$ en Tregs. Les Tregs inhibent la différentiation des cellules Th1 et Th2. Un sous-groupe Th17 mixte est induit par le TGF-β. Ces cellules expriment FoxP3 et RORγt et produisent à la fois IL-17 et IL-10. En absence de TGF-β, IL12 induit des cellules qui expriment des facteurs de transcription spécifique Tbet-RORγt. Ce sont des cellules IL-17-IFNγ libérant d'autres cytokines pro-inflammatoires.

3-3-4. Le TGF-β et le CHC

De nombreux articles ont mis en évidence une relation étroite entre l'augmentation des taux de TGF-β et le développement du CHC et proposent que le TGF-β puisse devenir un marqueur pronostique pour l'apparition de métastases intra-hépatiques (Hussein et al, 2012; Ito et al, 1991 ; Mamiya et al, 2010 ; Shirai et al, 1994). Le TGF-β participe au développement du CHC en induisant une TEM, mais il induit aussi la sécrétion de cytokines telles que le CTGF et favorise la croissance tumorale, l'angiogenèse, la migration et l'invasion cellulaire (Mazzocca et al, 2010) .

Pour mieux comprendre comment et par quels mécanismes le TGF-β induit la TEM au cours de la carcinogénèse hépatique, des études transcriptomiques ont été entreprises (Coulouarn et al, 2008; Fransvea et al, 2008 ; Mazzocca et al, 2010 ; Mazzocca et al, 2009). Ces études ont permis de mettre en évidence l'importance de la voie de signalisation TGF-β et d'identifier des groupes de gènes impliqués dans le développement du CHC. L'équipe de Coulouarn et al., a mis en évidence à partir des hépatocytes murins, une expression temporelle de gènes en réponse au TGF-β. Deux groupes de gènes ont été identifiés dans le développement du CHC ; un groupe représente les gènes impliqués dans la réponse rapide au TGF-β (signature rapide) et un deuxième correspond à la réponse tardive (signature tardive) (Coulouarn et al, 2008). Cette équipe a également montré que des patients atteints de CHC ayant une signature tardive avaient une survie plus faible que ceux ayant une signature rapide. L'identification de ces signatures n'est pas restreinte au CHC, et est aussi retrouvée dans le cancer du poumon où les tumeurs exprimant les gènes associés à la signature tardive sont corrélées à un caractère invasif (Coulouarn et al, 2008). De plus, Hoshida et al, ont intégré les données d'expression génique de neuf études différentes réalisées auprès de patients atteints de CHC. Dans ce modèle les gènes de la réponse au TGF-β sont associés à un mauvais pronostic de survie (Hoshida et al., 2009). L'ensemble de ces études met en évidence un lien entre l'expression des gènes du TGF- β et le phénotype invasif et agressif de la tumeur.

Des altérations de la voie de signalisation du TGF-β sont aussi observées dans certains CHC. En effet, des mutations de la protéine Smad2 surviennent dans 5% des cas de CHC, une perte de l'expression de la protéine Smad4 est retrouvée dans 10% des CHC (Longerich et al, 2004; Yakicier et al, 1999), et une réduction de l'expression du récepteur TβRII est observée dans 25% des CHC. Cette réduction d'expression est corrélée à un phénotype agressif et à l'apparition de métastases hépatiques (Mamiya et al, 2010).

Comme de nombreuses études ont montré une activation de la voie de signalisation du TGF-β au cours du CHC, l'inhibition cette signalisation semble être une piste thérapeutique intéressante. De fait, un essai clinique de phase 2 est en cours pour évaluer l'efficacité et la tolérance d'un traitement chez des patients ayant un carcinome hépatocellulaire, le LY2157299, un inhibiteur du récepteur TβRI qui bloque la phosphorylation des protéines Smads (essai clinique NCT01246986) (Bueno et al, 2008).

Des études *in vivo*, ont mis en évidence que l'expression de facteurs de transcription Snail et Twist chez des patients atteints de CHC est corrélée avec l'invasion tumorale et le développement de métastases (Lee et al, 2006 ; Sugimachi et al, 2003). Par ailleurs, le TGF-β joue un rôle clé dans la TEM notamment en partenariat avec d'autres protéines et d'autres voies de signalisation. Ainsi le TGF-β agit en synergie avec la laminine-5 ou avec d'autres facteurs de croissance comme le PDGF, pour induire une TEM complète des hépatocytes (Giannelli et al, 2005);(Fischer et al, 2007; Gotzmann et al, 2006).

Chapitre IV :

Effets du TGF-β sur les pathologies liées au VHC

4.1 La cytokine TGF-β et l'infection par le VHC

L'expression du TGF-β ou la perturbation de la voie de signalisation TGF-β sont impliquées dans les différentes étapes de l'hépatite C. Une étude de criblage double-hybride a mis en évidence plus de 300 interactions entre les protéines du VHC et les protéines cellulaires (de Chassey et al, 2008). Ces interactions perturbent de nombreuses voies de signalisation comme la voie de signalisation TGF-β . Cette perturbation provoque l'expression des protéines cellulaires qui peut réguler la synthèse, l'activation du TGF-β, les fonctions de nombreux acteurs de la voie de signalisation du TGF-β et la réplication du VHC.

4-2. Impact du TGF-β sur la réplication virale C

Plusieurs études indiquent que le TGF-β régulerait positivement la réplication du VHC. En effet, l'extinction du gène TGF-β réduit la réplication virale (Presser et al, 2011). Il a également été montré que le VIH pouvait avoir un rôle positif sur la réplication du VHC *via* un mécanisme dépendant du TGF-β (Lin et al, 2008). De plus, une étude récente décrit que le gène SNARK contribue à la réplication virale *via* la voie de TGF-β (Goto et al, 2013). L'ensemble de ces résultats suggère que le TGF-β aurait un rôle dans la réplication virale mais le mécanisme reste à éclaircir.

4-3. Influence du VHC dans l'induction de la fibrose du foie dépendant du TGF-β

L'infection par le VHC induit une inflammation chronique du parenchyme hépatique plus ou moins une stéatose, lésion dont les conséquences sont le développement de la fibrose. Plusieurs protéines virales semblent jouer un rôle dans le développement de la fibrose et de la cirrhose, plus particulièrement les protéines de la capside, NS3 et NS5A. En effet les protéines virales, NS3, NS5A et capside semblent jouer un rôle dans l'apoptose des hépatocytes, l'une des lésions élémentaires majeures de l'infection par le VHC (Schuppan et al, 2003). L'expression des protéines du VHC dans les hépatocytes contribue au développement de la fibrose hépatique en présence d'autres agents fibrogènes. En présence de CCl4, les souris transgéniques pour les protéines du VHC développent une fibrose hépatique plus sévère et présentent une perturbation du micro-environnement hépatique (Chouteau et al, 2012).

De nombreuses études dans les années 90 ont mis en évidence une augmentation de l'ARN messager du TGF-β ainsi que du TGF-β circulant chez les patients chroniquement infectés (Ito et al, 1991) ;(Shirai et al, 1994). Plusieurs études moléculaires montrent que l'expression accrue du TGF-β et de son taux circulant est corrélée avec le degré de fibrose hépatique

(Murawaki et al, 1998 ; Tsushima et al, 1999). En effet, des co-cultures d'hépatocytes infectés par la souche JFH-1 avec des CEH ou des $CD4^+$ ont permis de mettre en évidence que l'activation des CEH ou $CD4^+$ était médiée par l'expression augmentée de TGF-β par le virus (Schulze-Krebs et al, 2005), (Hall et al, 2010). De plus, la protéine de capside augmente la synthèse du CTGF, et exerce un rôle crucial dans la progression de la fibrose (Shin et al, 2005). Cependant, des taux élevés de TGF-β dans la MEC ne seraient pas la seule cause du développement de la fibrose. En effet, chez des patients chroniquement infectés, il a été mis en évidence des phosphorylations de Smad3 dans la région charnière par la protéine JNK. Ces phosphorylations dans la région charnière des R-Smads sont associées à la progression de la fibrose (Matsuzaki et al, 2007). En outre, l'augmentation de la fibrose induite par l'expression des protéines virales est notamment due à l'augmentation de la production de ROS qui peut être induite par plusieurs protéines virales comme la protéine de capside (Moriya et al, 2001) et NS5A (Gong et al, 2001). Le TGF-β est une cytokine dont le rôle est crucial dans le développement de la fibrose. La protéine de capside favorise également l'expression des métallo-protéases matricielles (MMP) 2 et 9 (Nunez et al, 2004), des protéines essentielles de la MEC dont l'augmentation est associée à la fibrogenèse (Han, 2006). Par ailleurs, des micro-RNA régulent aussi la voie de signalisation du TGF-β. De manière intéressante, l'augmentation de miR-21 interfère avec la voie de signalisation du TGF-β, régulant négativement Smad7 (Marquez et al, 2010). Chez des patients atteints d'hépatite C, une augmentation de l'expression de miR-21 est associée au stade de développement de la fibrose (Marquez et al, 2010). Le VHC diminuerait l'expression du miR-29 et dérégulerait la synthèse de la MEC par les CEH activées (Bandyopadhyay et al, 2011). Ainsi les miRNA pourraient jouer un rôle essentiel dans la fibrogenèse et deviendraient une nouvelle cible thérapeutique.

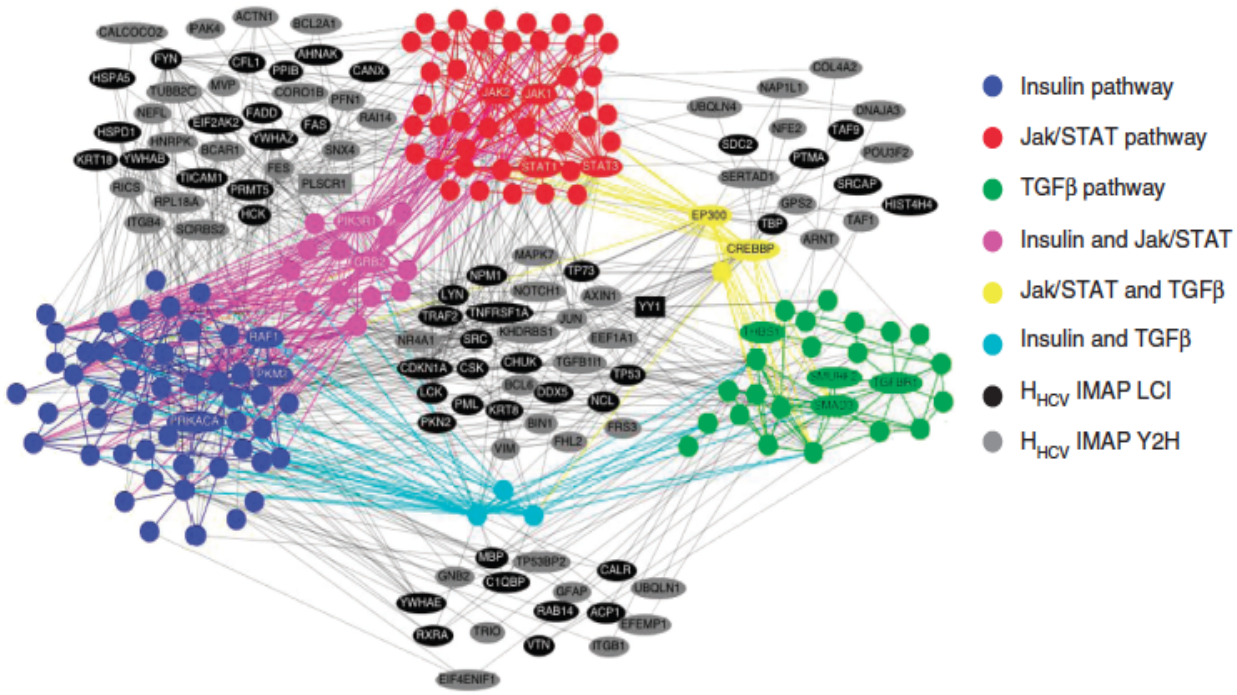

Figure 21 : Représentation des interactions entre les protéines virales C et les protéines humaines (de Chassey et al, 2008)

Interaction des protéines du virus de l'hépatite C avec différentes protéines cellulaires humaines impliquées dans plusieurs voies de signalisation.

4-3. La TEM chez les patients infectés par le VHC

L'inflammation chronique induite par l'infection virale VHC, représente une prédisposition majeure au développement d'un cancer du foie. Les mécanismes moléculaires associés au développement du CHC par le VHC sont encore mal compris. L'interaction entre les protéines virales et les protéines de l'hôte contribue probablement au processus oncogène viral (Fig.20). Quatre protéines virales pourraient jouer un rôle dans l'induction de la TEM : E1/E2, NS3-4A et NS5A (Akkari et al, 2012; Verga-Gerard et al, 2013; Wilson et al, 2012).

Le CHC est une tumeur très hétérogène. Dans le cas d'une hépatite virale, l'accumulation des protéines virales dans la cellule entraine un stress cellulaire qui favorise le développement d'altérations génétiques et l'accumulation de dommages dans l'ADN prédisposant les cellules à la mutagenèse (Tsai & Chung, 2010). Depuis quelques années, des études ont mis en évidence l'implication des protéines virales dans le développement du CHC, telles que les protéines d'enveloppes E1, E2, les protéines NS3, NS3-4A, NS5A (Akkari et al, 2012; Cheng et al, 2004 ; Choi & Hwang, 2006; Pavio et al, 2005). Les protéines d'enveloppe E1 et E2 sont impliquées dans le développement du CHC. Il a été montré que la protéine E2 pouvait induire la signalisation MAPK/ERK, favorisant ainsi la survie et la croissance cellulaire.

Wilson et al, ont mis en évidence que les glycoprotéines d'enveloppe, E1 et E2, augmentaient l'expression de Snail et de Twist favorisant ainsi le processus de la TEM dans les hépatocytes (Wilson et al, 2012). Des taux élevés de TGF-β circulant ont été retrouvés chez des patients chroniquement infectés par le VHC (Divella et al, 2012). Parmi les protéines virales, la protéine NS5A interagit avec le récepteur TβRI, ce qui produit une inhibition de l'apoptose induite par le TGF-β (Choi & Hwang, 2006). Cette interaction favorise également une TEM des hépatocytes primaires de souris due à une augmentation de l'expression de Twist2 mais aussi via l'interaction de NS5A avec l'oncogène Ras (Akkari et al, 2012). Une étude récente a mis en évidence que l'interaction des protéines NS3-4A avec la protéine Smurf renforçait la réponse cellulaire au TGF-β en terme de la TEM. Cette interaction promeut la TEM des cellules hépatocytaires infectées favorisant ainsi la progression tumorale (Verga-Gerard et al, 2013).

4-5. Interaction de la protéine de capside du VHC avec la cellule hôte

Outre sa fonction majeure de nucléocapside, plusieurs études indiquent que la protéine de capside est capable d'interagir avec plusieurs protéines cellulaires, et de modifier les fonctions biologiques comme la régulation de l'activité transcriptionnelle, la régulation du cycle cellulaire.

4-5-1. Influence de la protéine de capside dans l'induction de la stéatose hépatique

Plusieurs arguments suggèrent une relation étroite entre la protéine de capside et le métabolisme lipidique. En effet, *in vivo*, des souris transgéniques pour la protéine de capside du VHC de génotype 1b développent une stéatose (Moriya et al, 1997). Ces études montrent que la protéine de capside provoque une altération dans l'assemblage et la sécrétion des VLDL (Very Low Density Lipoprotein) dûe à une réduction de l'activité MTP (microsomal triglyceride transfer protein). Ce dysfonctionnement est en effet corrélé à la diminution des niveaux d'ApoB extracellulaires et de la taille des VLDL. Par ailleurs, l'interaction de la protéine ApoII avec la protéine de capside a été montré essentielle pour la sécrétion des VLDL car dans des souris doubles transgéniques déficientes pour la protéine ApoII et exprimant la protéine de capside une réduction de l'accumulation de la protéine de capside et de la production des VLDL est observée (Barba et al, 1997) (Perlemuter et al, 2002). De plus, la localisation subcellulaire de la protéine de capside à la surface des gouttelettes lipidiques serait en faveur d'une interaction avec la voie métabolique cellulaire. La protéine de capside

modifie la distribution intracellulaire des gouttelettes lipidiques en favorisant leurs déplacements sur les microtubules vers la périphérie du noyau cellulaire. Cette propriété permettrait l'interaction de la protéine capside avec les génomes viraux néo-synthétisés et les protéines non structurales, permettant ainsi la formation de la nucléocapside et l'empaquetage de l'ARN viral à partir des VLDL contenues dans ces gouttelettes lipidiques (GL) (Boulant et al, 2008). En outre la protéine de capside interagit avec la diacylglycérol acyltransférase-1 (DGAT1), une enzyme nécessaire dans la biosynthèse des GL qui conduit la protéine de capside à la surface de ces dernières et initie ainsi la production des particules virales (Herker et al, 2010). La protéine de capside joue un rôle important dans le développement de la stéatose car elle provoque l'altération de l'expression des gènes codant pour les protéines SREBP1 (Sterol regulatory element binding protein 1) et PPARg2 (Kim et al, 2007), impliquées dans la biosynthèse des acides gras qui contrôlent de nombreux aspects comme la prolifération cellulaire, la différenciation et le métabolisme des lipides (Tsutsumi et al, 2002). De plus La protéine de capside participerait aussi au développement de la stéatose en augmentant le niveau de stress oxydatif, en inhibant la β-oxydation et en induisant des dommages mitochondriaux (Okuda et al, 2002).

4-5-2. Influence de la protéine de capside sur les protéines suppresseurs de tumeurs

Des études mettent en évidence que la protéine de capside interagit avec des protéines régulatrices du cycle cellulaire comme p53, p73, pRb (Alisi et al, 2003 ; Cho et al, 2001; Lu et al, 1999 ; Ray et al, 1997). Par son interaction avec p53, la protéine de capside module l'expression de p21WAF1, une cible majeure de p53 et régule les activités des complexes de cycline kinases-dépendantes impliquées dans le contrôle du cycle cellulaire et la formation de tumeurs (Fig.21) (Yamanaka et al, 2002) (Lee et al, 2002) (Kwun & Jang, 2003). La protéine de capside module aussi l'expression de la protéine WNT1 favorisant de cette façon la croissance cellulaire (Fukutomi et al, 2005 ; Tsai & Chung, 2010). Elle pourrait aussi s'associer à l'extrémité cytoplasmique du récepteur de la lymphotoxine b et/ou au domaine intracellulaire du récepteur au TNF et moduler par ces voies les signaux d'apoptose (Zhu et al, 1998),

4-5-3. Influence de la protéine de capside sur la transcription des gènes

La protéine de capside a été décrite comme capable d'interagir avec des molécules de transduction dans le cytoplasme ce qui conduit à la modulation des transcriptions géniques dépendantes de ces voies de signalisation. En interagissant avec ces composantes cellulaires

comme Raf/MAPK (Aoki et al, 2000; Hayashi et al, 2000; Tsutsumi et al, 2003), β-Caténine (Levrero, 2006) la protéine de la capside permet la régulation, positive ou négative, des fonctions cellulaires (Delhem et al, 2001).

Dans le laboratoire, une étude a comparé les séquences de capsides isolées à partir d'hépatocytes obtenus par microdissection laser de nodules tumoraux ou cirrhotiques. L'analyse comparative de toutes ces séquences des variants isolés de zone tumorale ou non tumorale montre que ce gène de capside est fortement conservé. Par contre, l'analyse phylogénique révèle une distribution non aléatoire des variants de la capside en fonction des compartiments étudiés, montrant une compartimentation des quasi-espèces du VHC entre le foie tumoral et non tumoral. Nous avons émis l'hypothèse que les variants isolés de tumeurs pourraient avoir des propriétés biologiques différentes de ceux isolés de la zone non tumorale. En particulier, notre groupe a démontré que des variants isolés de tumeurs interagissaient avec Smad3 de façon plus importante que des variants isolés de zones non tumorales (Pavio et al.2005).

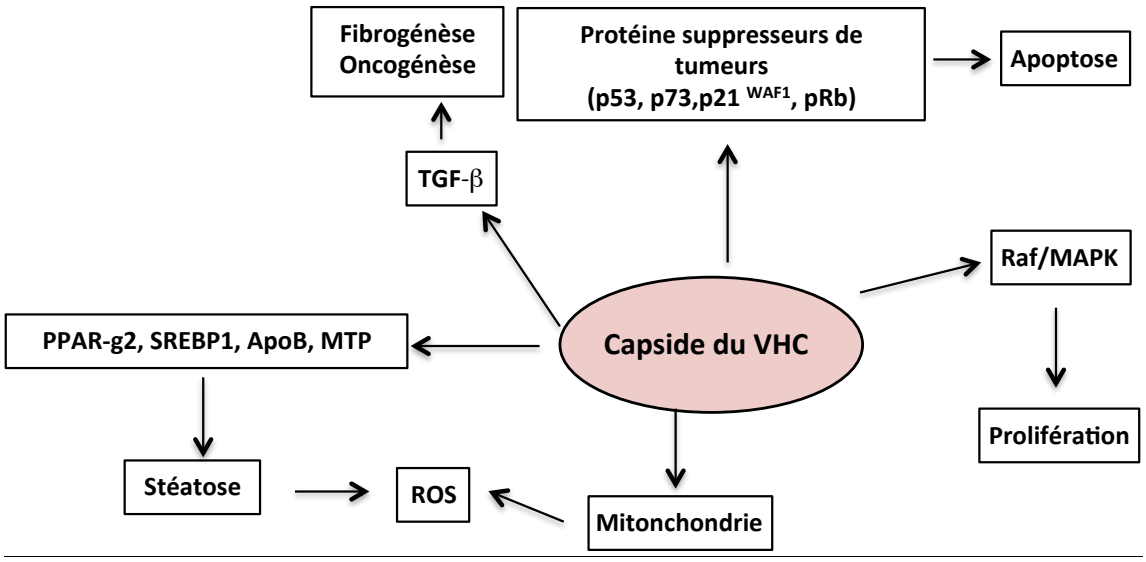

Figure 21 : **Conséquence de l'implication de la protéine de capside dans plusieurs voies de signalisation**

La protéine de capside est capable d'altérer des fonctions biologiques cellulaires en interagissant avec des protéines impliquées dans des voies de signalisations. L'interaction de la protéine de capside avec les protéines p53, P73 ou Raf perturbe la régulation

Objectifs

L'objectif de la thèse est de déterminer les mécanismes moléculaires de l'oncogenèse hépatique induite par le virus de l'hépatite C (VHC). L'hépatites virales C est un facteur de risque majeur du carcinome hépatocellulaire comme l'indiquent les études épidémiologiques, cependant les mécanismes moléculaires initiateurs de l'hépatocarcinogenèse par ce virus sont encore mal connus.

Les travaux du laboratoire ont précédemment permis de mettre en évidence dans des lignées d'hépatomes l'interaction de la protéine de capside du VHC avec la protéine Smad3, protéine majeure dans la transduction du signal TGF-β. Le TGF-β est une cytokine multifonctionnelle qui dans le contexte pathologique du cancer, joue un double rôle. Dans les étapes précoces de l'oncogenèse, le TGF-β contrôle la croissance tumorale en inhibant le cycle cellulaire et en induisant l'apoptose. En revanche, dans les étapes tardives, le TGF-β induit une transition épithélium-mésenchyme (TEM) qui favorise l'invasion cellulaire et le développement de métastases. L'ensemble de ces observations nous a conduits à élucider le rôle de la protéine de capside du VHC dans la transition épithélium-mésenchyme (TEM) induite par le TGF-β. Les travaux du laboratoire ont permis de mettre en évidence des quasi-espèces distinctes de la protéine de capside entre des hépatocytes tumoraux ou non tumoraux dans un même foie. Dans la continuité de ces travaux, nous avons déterminé les effets de ces variants de protéine de capside sur les réponses biologiques du TGF-β.

Mon travail de thèse est constitué de 2 axes de recherche. Dans un premier travail, j'ai étudié le cycle cellulaire ainsi que la TEM de cellules hépatocytaires exprimant différentes protéines de capside isolées à partir de tumeurs et de tissus non tumoraux en présence de TGF-β. J'ai ainsi identifié que l'expression de la protéine de capside déplaçait l'équilibre entre les effets suppresseur et promoteur de tumeur du TGF-β et qu'un variant isolé à partir d'une tumeur était plus efficace pour produire cet effet. Les résultats nous ont conduits à rechercher un rôle de la protéine de capside dans l'activation du TGF-β. L'ensemble de ces travaux nous a permis de proposer un modèle dans lequel le VHC pourrait contribuer au développement de la fibrose en augmentant l'activation du TGF-β et au développement du CHC en déplaçant les réponses biologiques de cette cytokine vers un effet promoteur de tumeur, stimulant ainsi la dissémination tumorale.

Nous avons dans un second axe de recherche identifié un nouveau marqueur de la transition épithélium-mésenchyme dans les hépatocytes. Celui-ci a été caractérisé *in vitro* sur des lignées d'hépatomes et validé *in vivo* sur les tissus hépatiques de patients atteints de carcinome hépatocellulaire d'origine diverse. L'expression de ce marqueur est corrélée à

l’expression de marqueurs progéniteurs L’expression de ce marqueur pourrait permettre de définir l'émergence de cellules subissant la TEM et sa capacité à générer de nouvelles cellules souches du cancer. Ce nouveau marqueur pourrait avoir une valeur pronostic importante dans le CHC.

Les résultats obtenus au cours de ces années de recherches seront présentés sous forme d’articles. Dans une dernière partie, je discuterai l’ensemble de ces résultats et proposerai les perspectives pour la suite de ce travail.

Résultats

Article I

« La protéine de capside du VHC déplace l'équilibre entre les effets suppresseurs et promoteurs de tumeurs du TGF-β. »

BATTAGLIA Serena, BENZOUBIR Nassima, NOBILET Soizic, CHARNEAU P, SAMUEL Didier, ZIGNENO AL, ATFI Aziz, BRÉCHOT Christian, BOURGEADE Marie-Françoise.

Liver cancer-derived hepatitis C virus core proteins shift TGF-beta responses from tumor suppression to epithelial-mesenchymal transition. Battaglia S, Benzoubir N, Nobilet S, Charneau P, Samuel D, Zignego AL, Atfi A, Bréchot C, Bourgeade MF. **PLoS One. 2009**;4(2):e4355. doi: 10.1371/journal.pone.0004355. Epub 2009 Feb 3.

L'hépatite chronique virale C et la cirrhose du foie représentent un facteur de risque majeur pour le développement du carcinome hépatocellulaire. Le VHC présente une hétérogénéité génétique importante qui se traduit par la présence d'une population virale hétérogène chez un même individu appelée quasi-espèces.

Une distribution différente des quasi-espèces de la protéine de capside du VHC isolée à partir d'un nodule tumoral (cT) ou non tumoral (cNT) a été observée. Il a également été montré que la protéine de capside interagissait avec la protéine Smad3, protéine majeure de la signalisation du TGF-β, et que la protéine de capside cT avait plus d'affinité pour Smad3 que la capside cNT. Nous avons alors émis l'hypothèse que ces protéines de capside pourraient avoir des effets différents sur les réponses biologiques aux TGF-β.

Pour étayer cette hypothèse, nous avons mené notre étude à partir de plusieurs systèmes cellulaires : des hépatocytes primaires humains et murins et des lignées d'hépatomes exprimant la protéine de capside T ou NT, nous avons montré que :

- Dans ces trois systèmes cellulaires, la protéine de capside diminue les effets anti-oncogéniques du TGF-β en terme d'arrêt de croissance et d'apoptose et au contraire augmente les effets promoteurs de tumeurs en terme de développement d'une TEM. Cet effet est plus important avec la protéine de capside cT.

- L'expression de la protéine capside et en particulier le variant cT induit une TEM en l'absence de TGF-β exogène. Cette TEM est reversée en présence d'un inhibiteur du récepteur du TGF-β suggérant que la protéine de capside active la signalisation du TGF- β.

- Des seuils différents d'activation de Smad3 sont nécessaires pour induire les effets anti ou pro tumoraux du TGF-β. En effet, de faibles niveaux d'activation de Smad3 sont suffisants pour induire une TEM par le TGF-β alors que des niveaux d'activation plus importants de Smad3 sont nécessaires pour induire l'apoptose.

Ces résultats suggèrent donc que la protéine de capside du VHC à travers sa liaison à Smad3 pourrait jouer un rôle important dans la carcinogenèse hépatique, en déplaçant l'équilibre entre les effets anti-tumoraux et les effets pro-tumoraux du TGF-β.

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PLoS one

Liver Cancer-Derived Hepatitis C Virus Core Proteins Shift TGF-Beta Responses from Tumor Suppression to Epithelial-Mesenchymal Transition

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Abstract

Background: Chronic hepatitis C virus (HCV) infection and associated liver cirrhosis represent a major risk factor for hepatocellular carcinoma (HCC) development. TGF-b is an important driver of liver fibrogenesis and cancer; however, its actual impact in human cancer progression is still poorly known. The aim of this study was to investigate the role of HCC-derived HCV core natural variants on cancer progression through their impact on TGF-b signaling.

Principal Findings: We provide evidence that HCC-derived core protein expression in primary human or mouse hepatocyte alleviates TGF-b responses in terms or growth inhibition or apoptosis. Instead, in these hepatocytes TGF-b was still able to induce an epithelial to mesenchymal transition (EMT), a process that contributes to the promotion of cell invasion and metastasis. Moreover, we demonstrate that different thresholds of Smad3 activation dictate the TGF-b responses in hepatic cells and that HCV core protein, by decreasing Smad3 activation, may switch TGF-b growth inhibitory effects to tumor promoting responses.

Conclusion/Significance: Our data illustrate the capacity of hepatocytes to develop EMT and plasticity under TGF-b, emphasize the role of HCV core protein in the dynamic of these effects and provide evidence for a paradigm whereby a viral protein implicated in oncogenesis is capable to shift TGF-b responses from cytostatic effects to EMT development.

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Introduction

Epithelial to mesenchymal transition (EMT) is defined as a process in which epithelial cells lose their phenotypic characteristic and acquire mesenchymal cell's features. While EMT is involved in the context of embryonic development it also plays a role in the genesis of fibroblasts during organ fibrosis in adult tissues and might contribute to the metastatic carcinoma development [1]. Indeed, EMT is being increasingly recognized as a crucial step that promotes cell migration, tumoral invasiveness and metastasis [2] and has also been implicated recently in cancer stem cell emergence [3]. In the liver, hepatic stellate cells (HCS) are considered as the major fibrotic precursor cells that transdifferentiate to fibrogenic, extracellular matrix producing myofibroblasts in inflammatory liver tissue upon TGF-b signaling, whereas hepatocytes undergo apoptosis upon signaling by this cytokine. However, identification of different fibrogenic populations apart of resident stellate cells [4] as well as convergent results of recent studies have challenged the paradigm of HSC as the essential source of liver myofibroblasts and inferred a prominent role for hepatocytes in liver fibrogenesis. Indeed, it has been reported recently that rat or mouse hepatocytes respond both in vitro and in vivo to TGF-b not only in terms of cell growth inhibition and apoptosis, but also in terms of induction of EMT [5–7]. Accordingly, it has been shown that TGF-b and laminin 5 transform non invasive hepatocellular carcinoma cells into invasive cells through induction of a complete EMT [8]. However, although the molecular mechanisms underlying EMT development have been studied extensively, little evidence is available concerning its physiological functions and relevance in human pathologies.

One of the mechanisms whereby cells undergo neoplastic transformation and escape from normal growth control involves an altered response to the cytostatic effects of TGF-b [9,10]. Furthermore, during the later stages of tumorigenesis, TGF-b can stimulate invasion mainly through induction of EMT. It is now generally accepted that TGF-b has a dual role in oncogenesis and can act as a tumor suppressor or tumor promoter factor depending on cellular context [11,12], but the mechanisms involved in the switch of TGF-b responses toward malignancy are not fully understood. In vivo, it has been shown that loss of TGF-b signaling significantly decreased tumor latency and increased the rate of metastasis in several mouse models [13].

TGF-β initiates responses by contacting two types of transmembrane serine/threonine kinases called receptors type I and type II, promoting activation of the type I by the type II kinase. The activated type I receptor then propagates the signal to the nucleus by phosphorylating Smad2 and Smad3. Once phosphorylated, Smad2 and Smad3 associate with the shared partner Smad4 and the complexes accumulate in the nucleus where they regulate the expression of TGF-β target genes through cooperative interactions with transcriptional partners [14,15,16]. Disruption of TGF-β signaling, either via mutational inactivation of components of the signaling pathway, or by modulation of their expression or function, is now known to play an important role in tumor progression. Despite all these evidences, the clinical implication of TGF-β in metastasis progression remains unclear.

Chronic hepatitis C virus (HCV) infection and associated liver cirrhosis represent a major risk factor for hepatocellular carcinoma (HCC) development, and despite epidemiologic evidence connecting HCV infection to HCC, the clinical impact of this virus on hepatocarcinogenesis is still unclear [17]. Because HCV RNA shows high genetic variability, chronic HCV infection results in a complex population of different but closely related viral variants commonly referred as quasispecies [18,19]. The non-random distribution of HCV quasispecies has been observed between tumoral and non-tumoral liver suggesting the possibility of a selection of quasispecies with modified functional properties that could contribute to fibrosis development as well as tumorigenesis process [20].

The structural component of HCV, HCV core protein has attracted particular attention after its characterization and various reports have suggested its potential role in HCV pathogenesis. Indeed, besides its role in viral RNA packaging, HCV core protein has been reported to interact with several cellular proteins such as TNFR [21], PKR [22], Stat3 pRB or p53 [23] leading to modulation of transcription of genes dependent on these cascades and consequently to modulation of a number of cellular regulatory functions. In fact, numerous data have suggested a possible involvement of HCV core protein in the modulation of cell proliferation and apoptosis although some results have been controversial given that core protein has been reported to exhibit pro or antiapoptotic effects depending on the experimental system used [24,25]. Moreover these studies were mainly performed using apoptotic agents from the TNF family and not with TGF-β. This discrepancy could also be due to genetic heterogeneity of different HCV genotypes.

We and others have previously demonstrated an interaction between Smad3 and the HCV core protein [26,27]. Interestingly, we also observed that different natural core variants isolated from tumor or non tumor nodules could differently bind Smad3, and consequently inhibit TGF-β induced Smad3 transcriptional activity suggesting that the HCV core protein may modulate TGF-β signaling and its downstream biological responses [27]. We hypothetised that the molecular heterogeneity of HCV observed in infected patients could be involved in the clinical course of cancer development.

Overexpression of TGF-β and concomitant decrease in hepatocyte growth inhibition is frequently observed in HCC supporting the notion that TGF-β could play a tumor promoting role in liver cancer [28]. However, the functional implication of TGF-β in liver tumorigenesis as well as the implication of EMT in HCC development are not yet elucidated. Likewise, effects of oncogenic viral hepatitis B or C proteins on EMT development have not been studied in the course of hepatocarcinoma process. Demonstrating interplay between HCV infection and TGF-β mediated EMT may provide a new model to gain insights in the mechanisms of liver carcinogenesis.

In this study, we made use of natural HCV core variants isolated from HCV-related HCC tissues to analyze their impact on the dual function of TGF-β in a pathophysiogically-relevant condition. Thus, we investigated the effects of core protein variants isolated from both tumor or non tumor cirrhotic areas in primary human hepatocytes; indeed, cirrhosis is a well-known preneoplastic condition, associated in at least 90% of cases of HCC. Using these variants we provide evidence for a paradigm in which a viral protein is capable to shift TGF-β responses from cytostatic effects to EMT development.

Materials and Methods

Materials

Recombinant TGF-β1 and recombinant TRAIL/Apo2L were purchased from Abcys, the chemical inhibitor of TGF-β signaling SB-431542 that acts by specifically interfering with the type I receptor [29] was from Calbiochem, the fluorescent dye $DiOC_6$ (3,3′dihexylocarbocyanine iodide) was from Molecular Probes.

Vectors

Full length HCV core sequences were amplified from HCV-RNA extracted from tumor (T) or cirrhotic (NT) nodules of a patient (patient B) infected with HCV 1b genotype as previously described [22]. PCR products were directly sequenced and inserted into the pcDNA3.1 vector. The sequence of these two variants has been previously described [27]. The T sequence differs from the NT one by 2 changes in aa 118 (N→D) and aa 189 (A→V).

$(CAGA)_9$-Luc was kindly provided by Dr J.M. Gauthier. The expression vectors for HA-TβRI.act, and Flag-TβRImL45.act were a gift from Dr. Y.E. Zhang [**30**]. The pRetroSuper-puro plasmid containing short hairpins RNA antisense against Smad3 was kindly provided by Dr J. Massagué [**31**]. A pRetroSuper-puro plasmid containing scramble short hairpins RNA was used as control. pIRES-GFP was obtained from Stratagene, pCMV-Renilla-luc was from Promega. Myc-Smad3 expression vector was previously described [32].

Transgenic mice

To obtain transgenic mice, the HCV core cDNAs isolated from tumor (T) or cirrhotic nodules (NT) were cloned downstream of hepatitis B virus regulatory elements and introduced into C57BL/6 embryos (Institut Clinique de la Souris, Strasbourg, France). Transgenic mice were identified by subjecting 1 μg of tail DNA to amplification by PCR.

Cell culture

The human hepatoma cell line Huh7 [33] was maintained in Dulbecco Modified medium containing 10% fetal calf serum (FCS). Cells were transfected with the different vectors using the LipofectAMINE method (Invitrogen) and stable transfectants were selected by incubating the cells with the antibiotic corresponding to the selection gene.

Isolation and culture of primary hepatocytes

Primary mouse hepatocytes were isolated by liver perfusion with a collagenase blend as previously described [34]. After isolation, hepatocytes were resuspended in Williams medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin, 250 ng/ml fungizone (“plating medium”) and plated at the density of 3×10^4 cells/cm^2. After 4 hours, serum-containing

medium was removed and cells were cultured in Williams medium supplemented with 1 mg/ml bovine serum albumin, 100 µg/ml streptomycin, 100 U/ml penicillin, 250 ng/ml fungizone, and treated with TGF-β 2 ng/ml or SB431542 1 µM.

Primary human hepatocytes were isolated from the healthy liver tissue of surgical liver biopsy specimens collected after informed consent obtained from patient undergoing therapeutic partial hepatectomy for liver metastasis and benign hepatic tumor. Collagenase (Sigma Aldrich) perfusion (500 µg/ml, 2.4 mg/ml $CaCl_2$ in HEPES buffer, pH 7.4) was preceded by extensive washing of the liver tissue with HEPES/EDTA buffer (pH 7.4) using a catheter inserted into the vessels on the cut surface of the resected fragment. Cells were then washed twice and hepatocytes were separated from nonparenchymatous cells by Percoll fractionation (30% isotonic Percoll solution, centrifuged at 450 g for 4 min) and immediately infected at 37°C for 2 h with lentiviral vectors, washed and plated in Williams medium supplemented as described elsewhere [35]. Twelve hours later, they were treated or not with TGF-β or SB431542 for various periods of time.

Lentiviral vectors

TRIP-ΔU3-CMV-T, TRIP-ΔU3-CMV-NT and TRIP-ΔU3-CMV-Cinv vectors were obtained by substituting GFP in TRIP-ΔU3-CMV-GFP with cDNA coding for HCV core sequences. An inverted core sequence TRIP-ΔU3-CMV-Cinv was used as a control.

Vector particles were produced by the transient calcium phosphate cotransfection of 293T cells as a previously described [36]. Vector concentrations were normalized according to the p24 (HIV-1 capsid protein) content of supernatants.

Western blotting

Cells were washed twice with PBS and lysed in RIPA buffer containing 0.5% SDS and Benzon nuclease. Proteins were quantified with the Bio-Rad protein assay (Bio-Rad, France) and 30 µg of extracts were separated on SDS polyacrylamide gel, transferred on nitrocellulose membrane and blotted using different primary antibodies directed against HCV core protein, E-cadherin, Fibronectin (Santa Cruz Biotechnology), Vimentin (Chemicon), phospho-Smad3 (Cell signaling), Smad3 (Abcam), Flag, Myc and HA tags (Sigma). Membranes were revealed using a chemioluminescence detection kit (ECL Plus, GE Healthcare).

Cell staining

Primary mouse hepatocytes were cultured for 48 h with or without TGF-β (2 ng/ml) and routine stain hematoxylin-eosin was performed after fixation of cells with EtOH 70% at 4°C for 15 min.

Immunofluorescence staining

Cells were washed with PBS and fixed with a 4% PFA solution at 4°C for 20 min followed by methanol permeabilization for 5 min at −20°C. Cells were then incubated with a primary mouse anti-vimentin, rabbit anti-αSMA, or rabbit anti-E-cadherin antibody and then with an Alexa Fluor 488 conjugated anti-mouse antibody and an Alexa Fluor 594 conjugated goat anti-rabbit antibody (Molecular Probes). They were then stained with Hoechst and examined by fluorescence microscopy.

Cell proliferation and apoptosis assays

Cell proliferation was assessed by BrdU incorporation (Roche), cell viability and caspase 3 activity were estimated using a Celltiter-Glo luminescent cell viability assay or the CaspaseGlo 3/7 assay respectively (Promega) according to the manufacturer's instructions.

Mitochondrial transmembrane potential ($\Delta\Psi m$) was evaluated by staining cells (10^6) with the fluorescent dye $DiOC_6$ at a final concentration of 40 nM for 15 min at 37°C. Cells were immediately dissociated by trypsin and their fluorescence estimated by analysis with a FACScan flow cytometer (Becton-Dickinson) using the FL1 channel [37].

Cell sorting

Flow cytometric analysis and sorting were performed using a FacsDiva flow cytometer (Becton Dickinson Immunocytometry Systems). Forward Scatter (FSC) and side scatter (SSC) were collected through a filter. The GFP signal was collected in the FL1 channel. A light gate was drawn in the SSC versus FSC to exclude dead cells/debris. Cells in the gate were displayed in a biparameter histogram (FS versus FL1) and final gating settings determined to collect the labeled cells. GFP positive cells were sorted at 5000 cells/sec.

Transcriptional analysis

Cells were cotransfected with vectors coding for the gene of interest together with the CAGA-luc reporter plasmid and the Renilla luciferase plasmid to normalize the results. They were incubated 24 h later in the absence or presence of TGF-β for another 18 h. Luciferase activity was measured with the Dual Luciferase reporter assay (Promega) system according to the manufacturer's instructions.

Statistical analysis

The significance between the different conditions and their control was determined by paired Student's *t* test using GraphPad Prism software. A p-value≦0.05 was considered significant.

Results

HCV core variants alleviate TGF-β cytostatic responses and increase TGF-β -mediated EMT in mouse or human primary hepatocytes

We have previously demonstrated that, when transiently expressed in hepatic cells, HCV core proteins isolated from tumor or cirrhotic nodules bind Smad3 differently and that this interaction inhibits Smad3-dependent transcriptional activity [27]. To ascertain the physiological relevance of this observation, we first investigated the impact of such binding on TGF-β biological responses in hepatocytes isolated from transgenic mice expressing these HCV tumor (T) or cirrhotic (NT) core variants under the control of the HBx promoter and which is mostly expressed in the liver. Hepatocytes were isolated from livers of 2 month old mice and treated or not with TGF-β for 48 hours. We observed that TGF-β was less potent to inhibit cell proliferation in hepatocytes isolated from transgenic mice expressing the HCV core proteins than in hepatocytes isolated from a control mouse (Fig. 1A). Accordingly, cell viability was less reduced by TGF-β in cells expressing the core proteins as compared to wild type cells (Fig. 1B). We also found that expression of the HCV core proteins inhibited TGF-β-mediated apoptosis as shown by caspase 3 activation, which represents a well-defined hallmark of apoptosis (Fig. 1C). Interestingly, T core expression decreased TGF-β -mediated apoptosis or inhibition of cell viability to a higher extent than the NT core showing a functional significance of the increased interaction of this core variant with Smad3 [27]. In order to verify that this HCV core-induced reduction of apoptosis

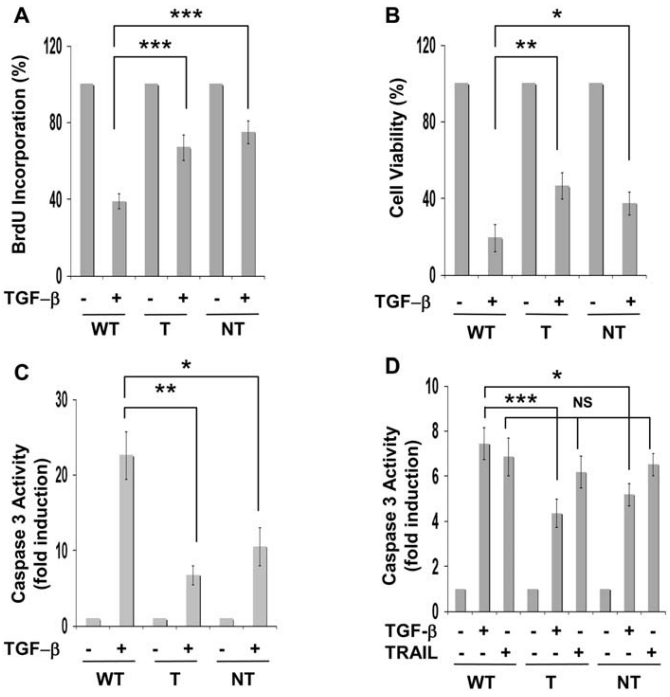

Figure 1. Expression of HCV core proteins in primary mouse hepatocytes reduce cell growth inhibition and apoptosis induced by TGF-β. (A,B,C) Mouse hepatocytes obtained from livers of transgenic mice expressing or not HCV core proteins isolated from tumor (T) or cirrhotic (NT) tissues were treated with TGF-β for 48 h before determination of cell proliferation, estimated by BrDU incorporation (A), cell viability (B) or caspase 3 activity (C). **(D)** Cells were treated with TRAIL (20 ng/ml) for 18 h before determination of caspase3 activity. Results represent the mean+/−SD of triplicates from a representative experiment. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$.
doi:10.1371/journal.pone.0004355.g001

observed after TGF-β treatment was specific, we used another inducer of apoptosis, TRAIL. Mouse hepatocytes expressing or not the HCV core proteins respond to TRAIL in a similar manner in terms of caspase 3 activation suggesting that the overall apoptosis process was not modified by core expression (Fig. 1D). This result is in agreement with a previous report indicating that HCV core leads to TRAIL-induced apoptosis through activation of the mitochondrial-signaling pathway [38].

Several lines of evidence support the notion that epithelial cancer cells lose their capacity to respond to TGF-β cytostatic effects but in some cases retain their ability to respond to other TGF-β -mediated functions such as EMT. The observation that HCV core proteins interfere with the ability of TGF-β to execute cell growth inhibition and cell killing prompted us to consider the possibility that these proteins might influence TGF-β mediated EMT. Since recent findings have demonstrated that TGF-β could induce an EMT in mature mouse hepatocytes *in vitro* [6,7], we investigated whether HCV core proteins could modulate the ability of TGF-β to promote EMT in the same primary hepatocytes. Contrast microscopy observation revealed that after treatment for 30 h with TGF-β some hepatocytes acquired a fibroblast-like morphology suggestive of EMT and that this effect was more pronounced when these hepatocytes express the core protein showing that cell plasticity could be increased in mouse hepatocytes expressing HCV core T protein (Fig. 2A). This observation was reinforced by videomicroscopy observation (data not shown). To confirm that these observed phenotypic changes were reflective of an EMT, we performed immunofluorescence analyses on hepatocytes isolated from control or from transgenic mice. In line with previous findings, TGF-β treatment of control mouse hepatocytes was accompanied by a very strong increase in the polymerization of the mesenchymal marker alpha smooth muscle actin (αSMA) consistent with a phenotype of EMT (Fig. 2B). Interestingly, HCV core proteins and particularly the T one could increase the αSMA fibers in the absence of exogenously added TGF-β. To assess whether autocrine release of TGF-β could be involved in the formation of αSMA stress fibers in HCV core expressing cells, we used a specific TGFβR1 inhibitor, SB431542. When these expressing cells were treated with this inhibitor, αSMA fibers completely disappeared, suggesting that the effect of the core protein on EMT development is mediated by an endogenous production of TGF-β (Fig. 2B). In accordance, Western blots analyses also showed that E-cadherin expression, an epithelial marker known to be lost in mesenchymal cells, was greatly decreased by TGF-β and fully restored by addition of SB431542 (Fig. 2C).

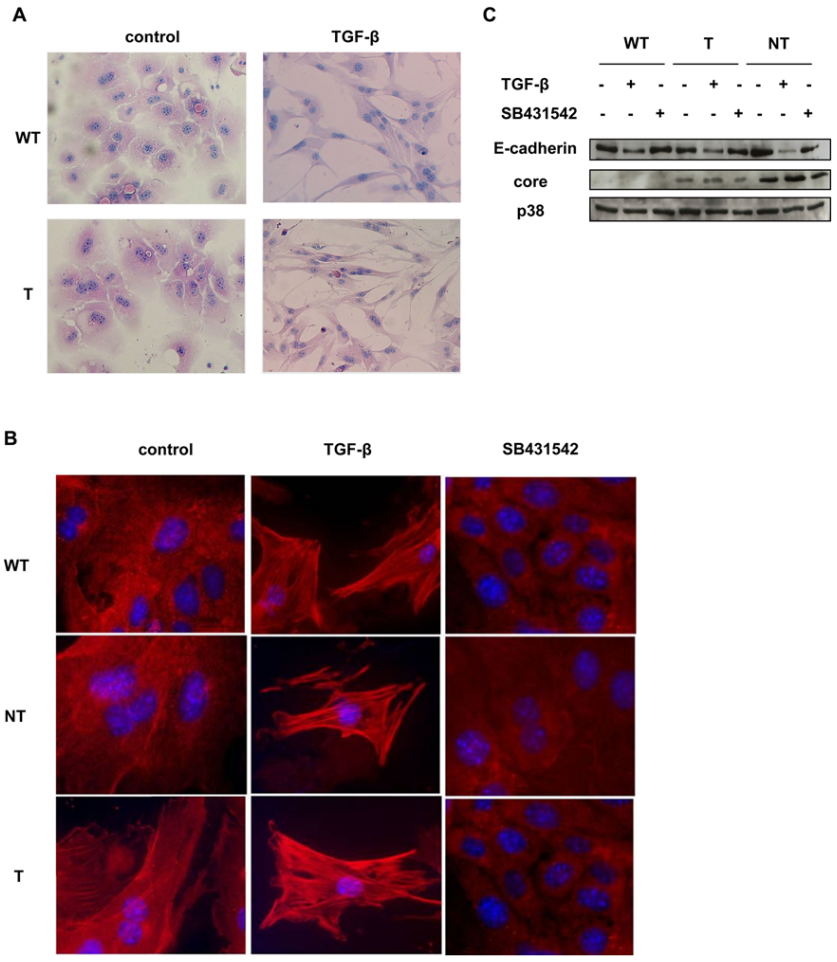

Figure 2. Expression of HCV core proteins in primary mouse hepatocytes increase EMT induced by TGF-β. (A) Morphologic changes of mouse hepatocytes expressing or not HCV T core protein observed after 48 h of culture with or without TGF-β (2 ng/ml). **(B)** Hepatocytes isolated from transgenic mice expressing HCV core proteins were treated with TGF-β (2 ng/ml) or SB431542 (1 μM) for 48 h and expression of αSMA was examined by immunofluorescence using a αSMA antibody. Data are representative of three independent experiments. **(C)** Hepatocytes isolated from transgenic mice expressing HCV core proteins were treated with TGF-β or SB431542 for 48 h and expression of E-cadherin was determined by Western blotting. Anti-p38 western blotting was used as control loading. Data are representative of three independent experiments.
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To obtain further evidence that HCV core proteins could modulate the magnitude of the negative growth regulatory effects of TGF-β we also performed experiments in human primary hepatocytes. Freshly isolated hepatocytes were infected with lentiviruses coding for the T or NT core variants or an inverted core sequence as control. Western blot analyses confirmed the expression of the core proteins (Fig. 3A). Cells were then treated or not with TGF-β for 96 h prior to analysis for cell viability or caspase 3 activation. Both TGF-β-mediated decrease in cell viability (Fig. 3B) and apoptotic responses (Fig. 3C) were alleviated by HCV core expression confirming the results obtained in mouse hepatocytes.

Although TGF-β -mediated EMT has been described in primary mouse or rat hepatocytes as well as in cancerous human cells, no such study has been yet investigated in primary human hepatocytes in vitro. Interestingly, we observed that human hepatocytes could express stress fibers as spikes mainly located in membrane protrusions under TGF-β treatment (Fig. 4A). Expression of HCV core proteins increased this TGF-β effect. Here again expression of the HCV core proteins increased αSMA

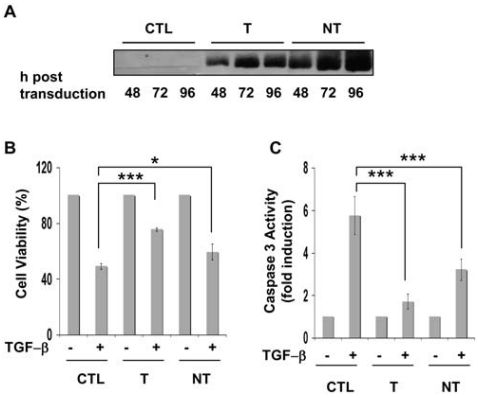

Figure 3. TGF-β cytostatic responses in primary human hepatocytes expressing HCV core proteins. Freshly isolated cells were infected with lentiviruses encoding the HCV core protein variants or an inverted core sequence as control (CTL) **(A)** Levels of core expression were estimated by Western blot analysis different time points after lentivirus transduction. **(B, C)** Determination of cell viability (B) or caspase 3 activity (C) was performed after 96 h of treatment with TGF-β (5 ng/ml). Results represent the mean+/−SD of triplicates from a representative experiment. * $p \leq 0.05$, *** $p \leq 0.0005$.
doi:10.1371/journal.pone.0004355.g003

polymerization even in the absence of exogenously added TGF-β. This effect could involve endogenous TGF-β since it was completely abolished in the presence of the TGF receptor inhibitor. To corroborate this result, we studied the expression of another mesenchymal marker, vimentin. In accordance with the data obtained with αSMA, we observed that in control hepatocytes vimentin expression was markedly increased after TGF-β treatment and that this increase was greater when hepatocytes expressed the NT core protein and even greater when T core was expressed (Fig. 4A). Similarly, core proteins induced vimentin expression and polymerization in the absence of exogenously added TGF-β. This expression was completely reversed by the TGFbRI inhibitor suggesting again that endogenously produced TGF-β could be responsible for this effect.

Western blots analyses evidenced a lower expression of E-cadherin after TGF-β treatment which was totally recovered in the presence of the TbRI inhibitor. On the contrary, expression of the mesenchymal marker fibronectin was greatly increased by TGF-β (Fig. 4B).

Taken together these data strongly suggest that HCV core interfere with TGF-β responses in terms of cell growth inhibition and apoptosis in hepatocytes isolated from transgenic mice as well as human primary hepatocytes. Remarkably, TGF-β responses, in terms of EMT are increased by expression of T or NT core protein variants in both mouse and human hepatocytes. This might reflect both direct effects of core on TGF-β-induced EMT and reduction of TGF-β induced apoptosis by the core protein, allowing more cells to undergo EMT as compared to control cells.

HCV core modulates TGF-β responses in Huh7 cells

In order to dissect the molecular mechanisms activated by the HCV core protein, we established Huh7 cell lines stably expressing the T core protein (Fig. 5A). Core protein inhibited TGF-β-mediated Smad3 transcriptional activity measured by expression in these cells of a reporter plasmid, which contains CAGA elements previously shown to be transactivated by TGF-β through Smad proteins (not shown). Consistent with the results observed in primary hepatocytes, we found that HCV core protein was able to decrease the inhibitory effect of TGF-β on cell viability (Fig. 5B). Similarly, TGF-β -mediated apoptosis was reduced in cells expressing HCV core as shown by caspase3 activation (Fig. 5C) or loss of mitochondrial membrane potential, which represents another early marker of apoptosis (Fig. 5D).

We then determined EMT process in Huh7 cell lines expressing this core protein. Immunofluorescence studies showed that αSMA was highly polymerized after TGF-β treatment associated with a strong decrease of E-cadherin from the cell membranes (Fig. 5E). αSMA polymerization was increased in core expressing cells. Interestingly, in the presence of core protein, αSMA fibers appeared even in the absence of exogenously added TGF-β. The expression of αSMA was accompanied with anchorage independent growth, which was observed in the absence of exogenously added TGF-β in HCV core protein expressing cells (data not shown).

All together, these data indicate that the effects of HCV core proteins on TGF-β responses observed in primary hepatocytes were reproduced in a human hepatoma cell line that could thus constitute an useful tool to dissect the mechanisms that are involved in the modulation of TGF-β responses.

We also compared protein core expression in our different cellular models and in extracts from liver of HCV/HCC patients. The strongest expression was obtained in human hepatocytes, which is consistent with an efficient lentiviral transduction. HCV core protein expression could be also detected in different liver extracts although at different levels. Interestingly, core expression in these extracts was comparable to the one observed in mouse hepatocytes (Fig. 5F).

Differential thresholds of Smad3 activation switch TGF-β responses from tumor suppression to tumor promotion

To analyze in more details the contribution of Smad activation in the effects of HCV core on TGF-β responses, we made use of a mutant of the TGF-β receptor I, TβRImL45Act that retains a constitutively active kinase domain but is unable to induce Smad phosphorylation. Huh7 cells were transfected with this mutant or with the wild type activated form of TβRI, together with a plasmid coding for the HCV core and GFP to detect the transfected cells. Immunofluorescence analysis was performed 48 h later. A marked polymerization of αSMA was observed through expression of the constitutively active TβR1 that was similar or even greater when cells also expressed the HCV core protein (Fig. 6A). This effect was completely lost when the cells expressed the TβR1 mutant thus demonstrating the need of activated Smads to initiate EMT.

To confirm this result, we established different independent Huh7 cell clones, stably expressing or not the HCV core protein, in which the expression of endogenous Smad3 was reduced by stable expression of a short-hairpin RNA (shRNA). As expected, Smad3 depletion prevented TGF-β -induced expression of the CAGA-luc reporter plasmid in the four independent clones tested, two of them expressing the core protein (Fig. 7B). Depletion of Smad3 also blunted the growth inhibitory and apoptotic actions of TGF-β (Fig. 7C and D). Smad3 inactivation also completely blocked TGF-β-induced EMT (Fig. 7E), further supporting the notion that Smad3 plays a crucial role in both tumor suppressor and pro-metastatic effects of TGF-β in carcinogenesis [39].

We next investigated the possibility that different threshold levels of Smad3 contribute to the differential effects of TGF-β on

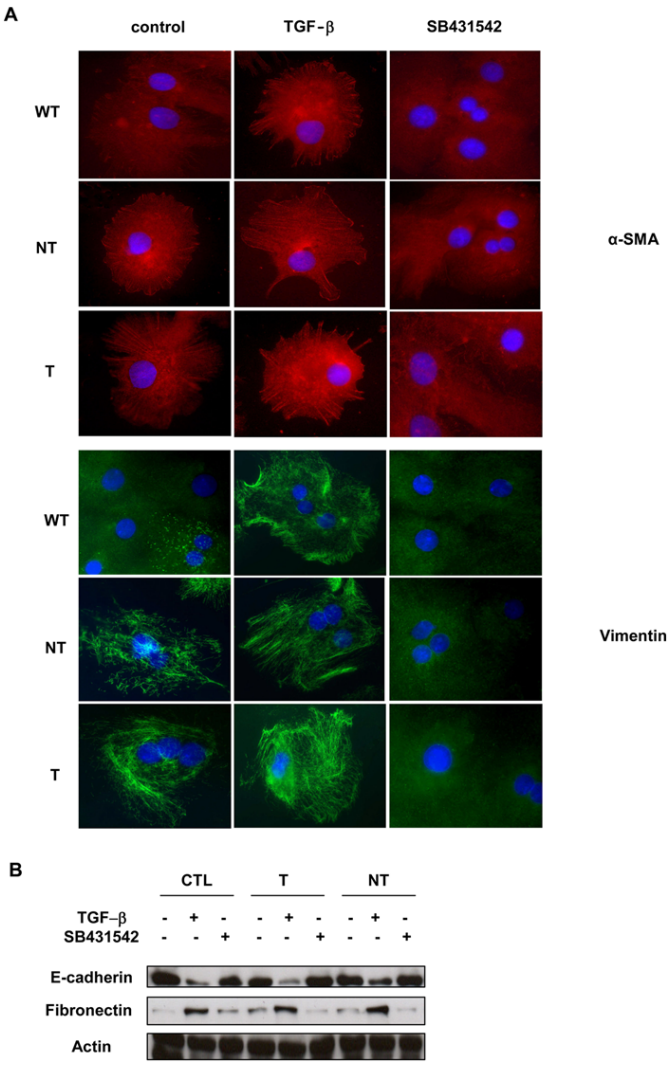

Figure 4. TGF-β increases EMT in primary human hepatocytes expressing HCV core proteins. (A) Expression of αSMA or Vimentin was estimated by immunofluorescence analysis after treatment with TGF-β (5 ng/ml) or SB431542 (1 μM). **(B)** Expression of Fibronectin or E-Cadherin was estimated by Western blot analysis in the same experimental conditions. Data are representative of three independent experiments.
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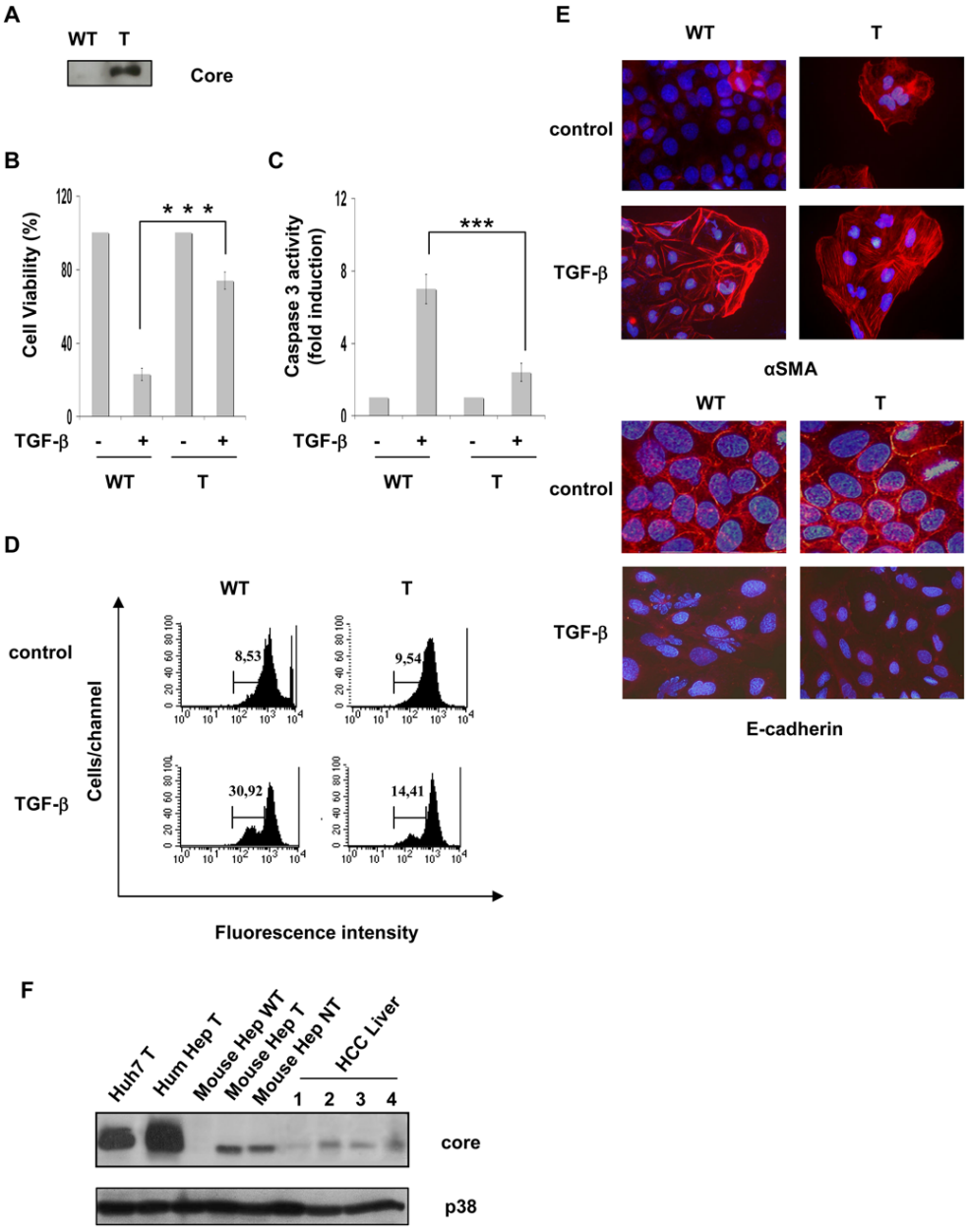
apoptosis or EMT. For this, we reintroduced increasing amounts of Smad3 in Huh7-shRNA-Smad3 clones (Fig 8A) and determined in these cells the levels of TGF-β signaling and anti-tumor or pro-tumor responses. As expected, in cells co-transfected with myc-Smad3 and CAGA-luc reporter plasmids, increasing Smad3 amounts resulted in the amplification of CAGA-luc transactivation after TGF-β treatment (Fig. 8B). Strong Smad3 expression led to consistent luciferase activity in the absence of TGF-β that may be due to constitutive Smad3 activation. To determine TGF-β responses in relation to Smad3 expression, Huh7-shRNA-Smad3 cells were also transfected with different amounts of myc-Smad3 plasmid, together with GFP plasmid and sorted on the basis of GFP expression prior to the addition of TGF-β. Interestingly, when Smad3 was weakly expressed, TGF-β induced apoptosis was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1 2 3 4
core
p38

Figure 5. TGF-β responses in Huh7 cells stably expressing HCV core protein. (A) Expression of HCV core protein determined by Western blot analysis. **(B, C)** Cells were treated with TGF-β (5 ng/ml) for 48 h before determination of cell viability (B) or caspase3 activity (C). Results represent the mean+/−SD of triplicates from a representative experiment. *** p≤0.0005 **(D)** Mitochondrial membrane potential (ΔΨm) was estimated by FACS analysis in cells treated with TGF-β for 48 h. After staining with $DiOC_6(3)$, cells with low fluorescence intensity corresponding to low (ΔΨm) were gated and their number expressed as a percentage of the total population. A representative experiment is shown. **(E)** Cells were treated with TGF-β for 48 h and E-cadherin or αSMA expression was assessed by immunofluorescence analysis. Data are representative of three independent experiments. **(F)** Comparative expression of HCV core proteins. Extracts from cultured cells expressing the core protein (Huh7, human or mouse primary hepatocytes) or from livers of HCV-related HCC patients were analyzed by Western blot. Anti-p38 western blotting was used as control loading.
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only marginal. In these experimental conditions, cell viability was comparable in control and TGF-β treated cells. When higher levels of Smad3 were expressed, decreased cell viability and increased apoptosis could be observed upon TGF-β addition. This is consistent with the notion that a high threshold of Smad3 is necessary to induce TGF-β -mediated anti-tumor responses (Fig. 8C and D).

The GFP positive cells were also analyzed for αSMA expression and polymerization after TGF-β treatment. In contrast with apoptotic data, TGF-β-induced EMT could occur in the context of low Smad3 expression (Fig. 8E). Taken together, these results strongly suggest that the amplitude of Smad3 activation may orientate TGF-β responses towards apoptosis or EMT. These observations could account for the induction of EMT by the HCV core protein despite diminution of TGF-β signaling.

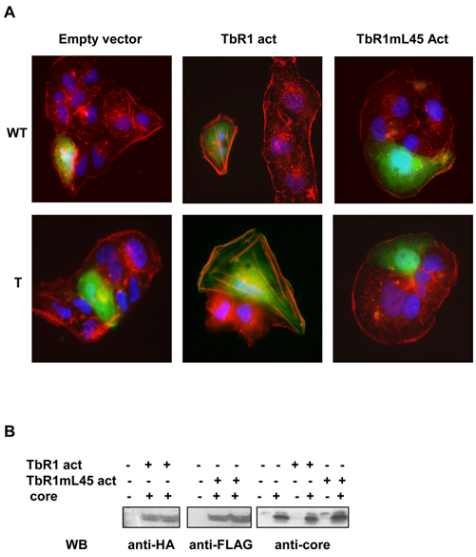

Figure 6. Smad activation is essential to induce TGF-β -mediated EMT. (A) Huh7 cells were co-transfected GFP together with TbR1act or TbR1l45M act plasmids in the presence or absence of HCV core vector. Immunofluorescence analysis was performed 48 h later with an anti αSMA antibody. **(B)** Expression of TbR1act or TbR1l45M act and HCV core protein were assessed by Western blotting using anti-HA, anti-Flag or anti-core antibodies respectively.
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Discussion

Our study offers relevant observations regarding both the mechanisms of HCV-related carcinogenesis and the impact of TGF-β in human cancer. Indeed, we provide evidence that HCC-derived HCV core proteins alleviate cell growth inhibition and apoptosis mediated by TGF-β indicating a biological significance of the binding of HCV core protein to Smad3. This effect was not restricted to stably transfected cell lines, since it was also observed in primary mouse hepatocytes isolated from transgenic animals expressing the core proteins as well as in primary human hepatocytes infected in vitro with lentiviruses encoding the same variants. Thus HCV core protein has also the potential to negatively impact the cytostatic actions of TGF-β in systems that may better reflect an *in vivo* situation. These data are in agreement with previous results suggesting that Smad3 is a predominant mediator of TGF-β-induced apoptosis. One attractive possibility could be that by interacting with Smad3, HCV core protein set a threshold level of TGF-β signaling that allowed for a modulation of the magnitude of TGF-β cytostatic responses. Consistent with this notion, we observed that overexpression of Smad3 could reverse this effect of HCV core on TGF-β responses in terms of Smad3 signaling, apoptosis and viability (not shown). Furthermore, this effect of HCV core protein on TGF-β cytostatic responses appears to be specific because it was not observed when another apoptotic cytokine such as TRAIL was employed.

Interestingly, in cells expressing HCV core proteins TGF-β was still able to reduce E-cadherin expression and increase αSMA expression and polymerization that are hallmarks of EMT. These alterations were associated with the ability of these cells to exhibit anchorage independent growth. Importantly, we also observed that core protein expression was sufficient to provoke EMT induction in primary hepatocytes. This effect was reverted by addition of a specific inhibitor of TGF-β I receptor thus demonstrating a TGF-β dependent effect of core on EMT development. . These data emphasize a differential effect on TGF-β actions in terms of apoptosis or EMT.

Different levels of HCV core expression have been observed in HCV-derived HCC at the mRNA level or in immunohistochemistry [40]. Using extracts isolated from livers of HCV/HCC patients we could detect core expression at the protein level. Moreover, we have previously shown that core protein extracted from HCV/HCC tumor tissue could bind Smad3 in GST-pull down analyses [27] suggesting that perturbation of TGF-β signaling may also be modulated in vivo. Overall these results are consistent with the hypothesis that this mechanism could operate during the development of HCV-induced HCC. Interestingly, both tumor and cirrhotic tissues-derived mutants demonstrated these biological effects; this likely reflects the preneoplastic nature of most cirrhotic nodules. However, we did observe a more pronounced biological effect of tumor-derived mutant on TGF-β signaling; this might suggest an HCV quasispecies selection in

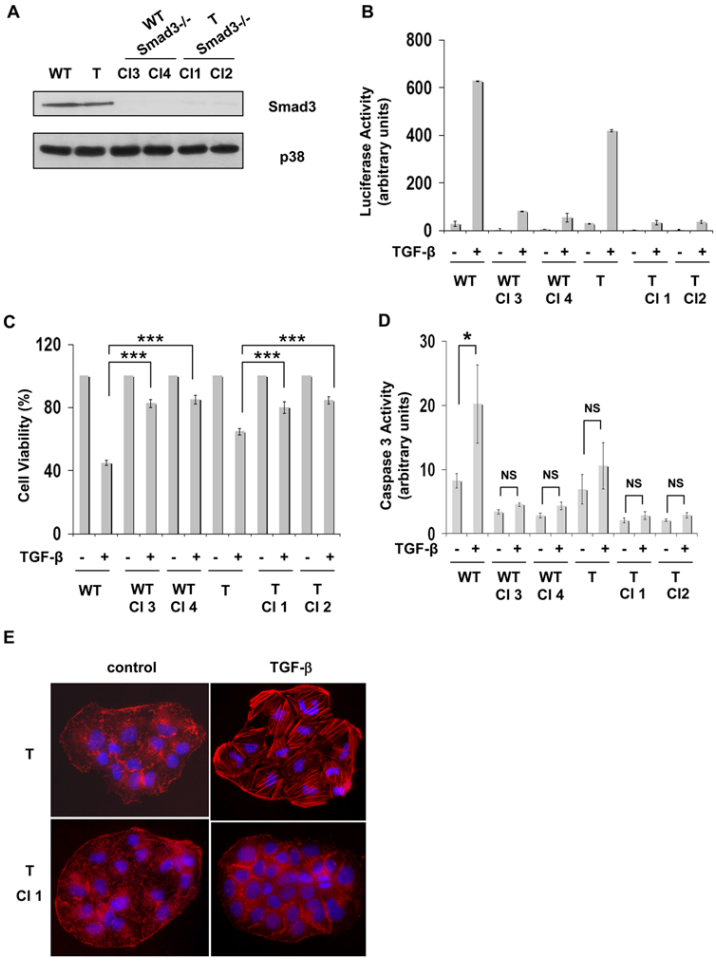

Figure 7. Smad3 depletion prevented TGF-β responses in Huh7 cells expressing or not the HCV core protein. (A) Smad3 expression determined by Western blot analysis in four independent clones selected after stable transfection with pRetroSuper-shRNA-Smad3 plasmid. Anti-p38 antibody was used as control loading. **(B)** Different clones were transfected with the CAGA-luc reporter plasmid and treated or not with TGF-β (5 ng/ml) for 18 h before determination of luciferase activity. Results were normalized with renilla luciferase and represent the mean of triplicates+/−SD. **(C, D)** Different clones were treated with TGF-β for 48 h before determination of cell viability (C) or caspase3 activity (D). Results represent the mean+/−SD of triplicates from a representative experiment. * p≤0.05, *** p≤0.0005, NS : not significant. **(E)** Different clones were treated with TGF-β for 48 h and αSMA polymerization was estimated by immunofluorescence analysis.
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clonally proliferating tumor cells, consistent with our previous analyses [20,22].

It is commonly accepted that TGF-β signaling pathway plays a tumor suppressor role thought to be associated with growth inhibitory and apoptotic responses and a tumor promoter role thought to reflect the positive effects of TGF-β on tumor cell invasion. Taken together, our data suggest that HCV core, by reducing Smad3 signal strength, renders the cells to become less sensible to tumor suppressive effects of TGF-β although they retain the tumor promoting effects, assuming that Smad3 may regulate different targets in function of its level of activation. This is consistent with the notion that critical signal amplitude may be needed to evoke a biological effect. In addition to Smad pathways, non-smad-dependent signal transduction downstream of TGF-β receptors has been proposed [41,42]. Among them, the MAP Kinase pathways including ERK, JNK or p38 as well as PI3K/AKT have been shown to be modulated by TGF-β. Since different reports have shown that HCV core protein could also modulate

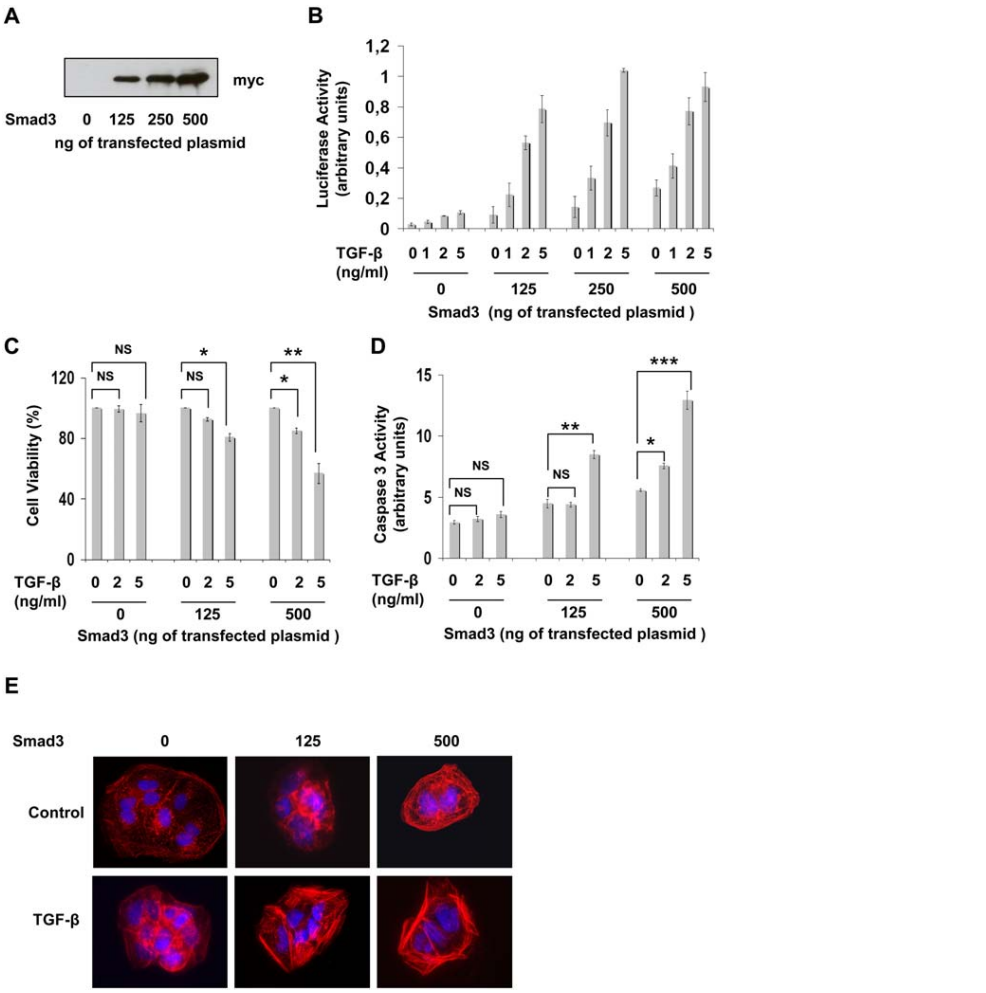

Figure 8. TGF-β responses in Huh7 cells expressing different levels of Smad3. (A) Huh7-shRNA-Smad3 cells (Clone 3) were transfected with increasing amounts of Myc-Smad3 expression vector together with pCMV renilla luciferase. Smad3 protein expression was evaluated 24 h later by Western blot analysis using an anti-Myc antibody and loading was normalized with renilla luciferase expression. **(B)** Huh7-shRNA-Smad3 cells (Clone 3) were cotransfected with the CAGA-luc reporter plasmid and increasing amounts of Myc-Smad3 vector together with pCMV renilla luciferase. 24 h later, they were treated or not with different doses of TGF-β for 18 h before determination of luciferase activity. Results were normalized with renilla luciferase and represent the mean+/−SD of triplicates from a representative experiment. **(C, D)** Huh7-shRNA-Smad3 cells (Clone 3) were transfected with increasing amounts of Myc-Smad3 vector together with pGFP plasmid and sorted by FACS 24 h later on the basis of GFP expression. Cells were then cultured for 24 h and treated with different doses of TGF-β for 48 h before determination of cell viability (C) or caspase3 activity (D). * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$, NS : not significant. **(E)** Huh7-shRNA-Smad3 cells (Clone 3) were transfected with increasing amounts of Myc-Smad3 vector together with pGFP plasmid and sorted 24 h later on the basis of GFP expression. αSMA expression was estimated by immunofluorescence analysis after treatment with TGF-β (1 ng/ml) for 48 h.
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these pathways, alternative mechanisms could also contribute to TGF-β responses leading to tumor promotion.

It has been recently reported that hyperactive Ras mediates a decrease in TGF-β -induced Smad3 phosphorylation in the COOH-terminal and an increase in JNK-induced Smad3 phosphorylation in the linker region, shifting the TGF-β pathway from a tumor suppressive to an invasive capacity in human colorectal as well as hepatic carcinogenesis [43]. Using a

different model, our results, relevant for human carcinogenesis, show that reduction of Smad3 activation could account for a tumor promoting role of TGF-β and raise the possibility that core protein may trigger one step of liver carcinogenesis by modulating the balance between TGF-β antitumor or protumor responses.

Although activation and transdifferentiation of hepatic stellate cells are still regarded as key mechanisms of fibrogenesis, recent studies have pointed out that other liver cells, including hepatocytes may contribute to the pool of myofibroblasts in fibrosing liver. Our results showing that TGF-β is able to induce EMT in primary mouse and human hepatocytes add further evidence for this concept. Furthermore, because HCV replicates in hepatocytes, the fact that EMT could develop in HCV core-expressing cells under TGF-β might provide a new notion to explain the fibrotic effect of this virus.

In conclusion, our data ties together TGF-β and HCV which are both known to be keys in the development of fibrosis and HCC, highlight the ability of hepatocytes to develop EMT under TGF-β and emphasize the role of HCV core protein in the dynamic of these effects.

Moreover, one report has suggested EMT as a mechanism of Epstein-Barr virus-related tumor cell dissemination [44]. Adenoviruses [45] or Papilloma viruses proteins have also been reported to interact with Smad3-dependent transcription [46,47]. Overall, our present results significantly reinforce the hypothesis that TGF-β and EMT are important drivers of virus-induced human cancers.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: SB CB MFB. Performed the experiments: SB NB SN MFB. Analyzed the data: SB DS ALZ AA CB MFB. Contributed reagents/materials/analysis tools: PC. Wrote the paper: SB CB MFB.

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Article II

« La protéine de capside du VHC induit l'activation du TGF-β latent *via* l'expression de la Thrombospondine. »

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HCV core-mediated activation of latent TGF-β via thrombospondin drives the crosstalk between hepatocytes and stromal environment. Benzoubir N, Lejamtel C, Battaglia S, Testoni B, Benassi B, Gondeau C, Perrin-Cocon L, Desterke C, Thiers V, Samuel D, Levrero M, Bréchot C, Bourgeade MF**. J Hepatol. 2013** Dec;59(6):1160-8. doi: 10.1016/j.jhep.2013.07.036. Epub 2013 Aug 6

Le TGF-β joue un rôle majeur dans le développement des pathologies hépatiques principalement par ses effets sur la réponse immunitaire, la fibrose et la cancérogenèse. C'est une cytokine produite sous forme d'un complexe latent sécrété dans la matrice extra-cellulaire (MEC). L'activation du TGF-β est une étape clé pour induire une signalisation et une réponse cellulaire du TGF-β. L'activation du TGF-β latent peut se faire selon plusieurs mécanismes qui aboutissent à la libération du TGF-β actif.

Précédemment nous avons montré que les variants de la protéine de capside du VHC isolées à partir d'une région tumorale (cT) et non-tumorale (cNT) sont capables de modifier l'équilibre des réponses biologiques du TGF-β, en faveur de la promotion tumorale. De manière intéressante, nous avons remarqué que la protéine de capside du VHC est capable d'induire une TEM en absence de TGF-β exogène et que cet effet est reversé par l'addition d'un inhibiteur spécifique du récepteur du TGF-β. De ce fait, nous avons déduit que la protéine de capside pourrait de façon directe ou indirecte activer le TGF-β latent.

Pour soutenir cette hypothèse, nous avons mené l'étude à partir de plusieurs lignées cellulaires exprimant soit, les protéines de capside (cT, cNT), le réplicon du VHC, les protéines non structurales du VHC ainsi que des hépatocytes primaires humains et des hépatocytes primaires murins exprimant la protéine de capside cT ou cNT. Nous avons montré que

- 50% des gènes dérégulés par la protéine de capside sont des gènes cibles du TGF-β. Ceci a été observé à partir d'une étude transcriptomique réalisée avec des hépatocytes de souris transgéniques pour les capsides cT ou cNT.

- la protéine de capside active le TGF-β latent. En effet, nous avons quantifié du TGF-β actif dans des foies de souris transgéniques pour les protéines de capside mais aussi dans les surnageants de culture des cellules exprimant les différentes protéines de capside traitées au TGF-β latent. De plus, nous avons montré que la signalisation du TGF-β était activée dans ces mêmes cellules.

- le TGF-β activé par la protéine de capside active les cellules étoilées en myofibroblastes lors d'une co-culture de ces dernières avec des hépatocytes exprimant les différentes protéines de capside.

- Par l'étude transcriptomique nous avons identifié que la protéine de capside induisait l'expression de la thrombospondine, protéine connue pour activer le TGF-β latent. L'expression de cette protéine a été observée dans le milieu de culture des hépatocytes primaires murins et de la lignée d'hépatome.
- La protéine de capside active le TGF-β latent *via* la thrombospondine. En effet, des expériences montrent qu'en présence d'ARN interférant pour la thrombospondine, des cellules hépatome exprimant la protéine de capside cT ou cNT ne sont plus capables d'activer le TGF-β latent exogène et d'induire une signalisation TGF-β dans ces cellules.

Ces résultats définissent un nouveau concept où le VHC, à travers la protéine de capside pourrait contribuer au développement de la fibrose en augmentant l'activation du TGF-β et de ce fait en activant les cellules étoilées par un mécanisme paracrine. Il contribuerait au développement du CHC en déplaçant les réponses biologiques de cette cytokine vers un effet promoteur de tumeur.

Research Article

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HCV core-mediated activation of latent TGF-β via thrombospondin drives the crosstalk between hepatocytes and stromal environment

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Background & Aims: The mechanisms by which fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) develop during chronic hepatitis C virus (HCV) infection are not fully understood. We previously observed that HCV core protein induced a TGF-β-dependent epithelial mesenchymal transition, a process contributing to the promotion of cell invasion and metastasis by impacting TGF-β1 signalling. Here we investigated HCV core capacity to drive increased expression of the active form of TGF-β1n transgenic mice and hepatoma cell lines.

Methods: We used an *in vivo* model of HCV core expressing transgenic mice.

Results: We observed that about 50% of genes deregulated by core protein expression were TGF-β1 target genes. Active TGF-β levels were increased in HCV core transgenic mouse livers. Overexpression of core protein in hepatoma cells increased active TGF-β levels in culture supernatants and induced Smad2/3 phosphorylation, thus reflecting activation of the TGF-β signaling pathway. Moreover, our data showed the implication of thrombospondin-1 in core-dependent TGF-β activation. Finally, hepatoma cells expressing HCV core could activate stellate cells in co-culture and this activation was TGF-β dependent.

Conclusions: Collectively, these data delineate a novel paradigm where HCV may be related to liver pathogenesis through its ability to induce a local, intrahepatic TGF-β activation. They argue for a dual impact of HCV core on liver fibrosis and liver carcinogenesis: HCV core could act both as autocrine and paracrine factor modulating TGF-β responses within hepatocytes and in stromal environment through TGF-β activation.

Keywords: HCV core; Active TGF-beta; Stellate cell activation; Paracrine effect.
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Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; EMT, epithelial mesenchymal transition; TGF-β, transforming growth factor beta; ECM, extracellular matrix; TSP, trombospondin; cT, HCV core from tumor nodule; cNT, HCV core from non-tumor nodule; SMA, smooth muscle actin; HSC, hepatic stellate cell.

Introduction

Chronic hepatitis C virus (HCV) infection is associated with a major risk of developing progressive liver diseases including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [1]. Despite epidemiologic evidence connecting HCV infection to HCC as well as a large body of literature from clinical and animal studies, little is known about how HCV causes fibrosis and subsequent development of cirrhosis and HCC [2]. The long latency period between HCV infection and HCC may signify indirect action of this virus. It could be postulated that persistent stimulation of cellular stress responses by viral proteins within hepatocytes may modulate the microenvironment that plays an important role in chronic infections. It is thus of interest to obtain data that provide information about host proteins of predicting importance in HCV-related liver diseases since different reports highlight the crosstalk between infected cells and the surrounding stroma as a key modulator of the processes of fibrosis, epithelial mesenchymal transition (EMT), tumor invasion, and metastasis. Several lines of investigation pointed out that, besides its role in viral RNA packaging, HCV core protein interacts with several cellular proteins leading to modulation of transcription of genes dependent on these cascades and consequently to modulation of a number of cellular regulatory functions [3] including

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control of cell growth, apoptosis, oxidative stress, carcinogenesis, and immune modulation.

Overexpression of TGF-β and concomitant decrease in hepatocyte growth inhibition are frequently observed in HCC, supporting the notion that TGF-β could play a tumor promoting role in liver cancer [4]. TGF-β is a multifunctional cytokine involved in the regulation of immune response, cell cycle, differentiation, and apoptosis. TGF-β acts as a two-edged sword in oncogenesis acting as a tumor suppressor or promoter depending on the stage of tumorigenesis [5]. Moreover, in the inflammatory liver, hepatic stellate cells transdifferentiate into fibrogenic, extracellular matrix (ECM) producing myofibroblasts upon TGF-β1 signaling [6].

TGF-β1 is secreted as a latent complex that is targeted to the extracellular matrix. This latent complex is composed of mature TGF-β1, latency associated protein (LAP), and latent TGF-β binding protein (LTBP) [7]. Activation of latent TGF-β1nvolves disruption of the interaction between LAP and mature TGF-β to permit binding of the cytokine to its receptor and subsequent activation of transcriptional responses through the specific Smad pathway.

Extensive work on this process led to the identification of two classes of TGF-β activators: various proteases could activate TGF-β by degrading LAP, and other proteins such as integrins [8] and thrombospondin-1 (TSP-1) [9] were shown to alter LAP conformation allowing TGF-β1 activation. Just a minor fraction of total TGF-β produced by the cells is made available for signaling, suggesting that the balance between latency and activation represents a major regulatory step to set strength and precise localization of TGF-β activity within tissues [10]. Although clinical and experimental studies have demonstrated that TGF-β1 levels were increased in chronic HCV infection [11,12], no study has yet determined whether the balance between latent and active TGF-β levels has been modulated during chronic HCV infection.

We previously reported that HCV naturally occurring variants of HCV core isolated from tumoral and non-tumoral liver of the same patient were able to shift TGF-β1 responses from tumor suppressor effects to tumor promotion by decreasing hepatocyte apoptosis and increasing EMT through a decrease of Smad3 activation. Moreover, we observed that core protein expression was sufficient to provoke EMT induction in primary hepatocytes. This effect was reverted by addition of a specific inhibitor of the TGF-β1 receptor suggesting a TGF-β1 dependent effect of core on EMT development [13,14].

We here report that HCV drives activation of TGF-β through HCV core. Indeed, we show that mouse primary hepatocytes obtained from mice transgenic for two different core sequences or HuH7 cells stably expressing these cores exhibit a permanent activation of the TGF-β pathway and are able to activate hepatic stellate cells in co-culture.

Cumulatively, our data describe a novel paradigm for the role of a viral protein in liver fibrosis and HCC where HCV core acts both by modulating TGF-β responses in hepatocytes in an autocrine mechanism and by affecting the stellate cell activation in a juxtacrine/paracrine mechanism through TGF-β activation.

Materials and methods

Materials

Active TGF-β1 was from Abcys, and latent TGF-β1 from R&D. The TGF-β receptor inhibitor SB431542 was from Calbiochem, Leu-Ser-Lys-Leu (LSKL) peptide was from AnaSpec.

Vectors

See Supplementary Material.

Cells

HuH7, HuH7.5, FL/neo Replicon cells [15], and HuH9.13 cells expressing the HCV NS3-NS5B subgenomic replicon [16] were maintained in DMEM containing 10% fetal calf serum. HuH9.13-cured cells were obtained from HuH9.13 after 1-month treatment with 500 U/ml interferon α2a. The human stellate cell line LX-2 [17] was maintained in DMEM containing 2% fetal calf serum. Cells were transfected with the different vectors using X-treme (Roche) or siRNA using jetPRIME (Polyplus). Primary hepatocytes from mice transgenic for HCV core were isolated by *in situ* collagenase perfusion of livers as previously described [14].

HuH7.5 infection with JFH1

High titer stocks of cell culture–derived JFH1 were prepared as described elsewhere [18]. HuH7.5 cells were seeded in 6-well plates and inoculated 24 h later with increasing concentrations of cell culture–derived JFH1 (0.5, 1, 5, 20, and 50 HCV mRNA copies/cell). After 16 h at 37 °C, the inoculum was removed and cells were washed three times with DMEM. Two ml/well of fresh medium was then added. Cell supernatant was collected 72 h post-infection and harvested for HCV RNA evaluation or protein analysis by Western blotting. Usually, in these experimental conditions, 60–80% of cells are infected when 50 RNA copies/cells are applied (i.e., HCV CORE or NS5A positive cells).

Microarrays, Taqman® Low Density Arrays (TLDAs) and qRT-PCR

See Supplementary Material. Data deposition is in Gene Expression Omnibus (GEO) under GEO Accession GSE36220 at http://www.ncbi.nlm.nih.gov/geo/.

Western blotting

Cells were lysed in RIPA buffer containing 0.5% SDS and Benzon nuclease (Novagen). Proteins were quantified and extracts separated on SDS polyacrylamide gel, transferred on nitrocellulose membrane and blotted with different antibodies. Membranes were revealed with a chemioluminescence detection kit (ECL Plus, GE Healthcare) using a DCC camera (G Box Syngene).

Immunofluorescence staining

Cells fixed with a 4% PFA solution, permeabilized with PFS (saponin gelatin in PBS) were first incubated with an anti-αSMA antibody (Clone 1A4, Sigma), and after with an Alexa Fluor 488 conjugated anti-mouse antibody (Molecular Probes). They were counterstained with Hoechst and examined by fluorescence microscopy.

Luciferase reporter experiments

Cells were co-transfected with vectors coding for the gene of interest together with FoxH1 and ARE-luc (Activin Responsive Element) or SBE-Luc (Smad Responsive Element) reporter plasmids and the *Renilla* luciferase plasmid to normalize the results. They were incubated 24 h later in the absence or presence of TGF-β1 or SB431542 for another 18 h. Luciferase activity was measured with the Dual-Luciferase Reporter Assay (Promega).

Determination of active TGF-β1 levels

See Supplementary Material.

Statistical analysis

Statistical differences between conditions were validated by paired *t* test with significant $p < 0.05$.

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Results

HCV core induces TGF-β1 target gene expression

Overexpression of the HCV core protein has been shown to modulate multiple intracellular signaling pathways, including TGF-β1, and to broadly affect gene expression in both hepatoma cell lines and primary hepatocytes [19]. In order to get a more comprehensive insight into the consequences of core-mediated deregulation of SMAD-mediated signaling on gene expression and TGF-β1 target genes, we analyzed global expression profiles using the Mouse Gene 1.0 ST Affimetrix microarrays. Primary hepatocytes were isolated from transgenic mice expressing either non-tumoral, cNT or tumoral cT and their control littermates (Supplementary Fig. 1) cultured for 24 h and then treated with TGF-β1 for 2 h before total RNA extraction. Out of 35,500 transcripts present in the array, 217 were significantly modulated when TGF-β1 treated cells were compared to their respective controls. TGF-β1 regulated genes could be further classified on the basis of their differential regulation in control or HCV core expressing hepatocytes (Fig. 1A). Comparison of this gene signature with published gene expression data sets [20] revealed that 75% of these genes were previously described as TGF-β1 target genes in mouse hepatocytes. This level of overlap supported a robust TGF-β1 signature in our transcriptomes.

This Venn diagram allowed us to design a customized Taqman® Low Density Array (TLDA) focused on genes regulated by core. Consequently, 84 genes modulated in control or TGF-β1 treated cNT or cT were selected.

Real-time PCR analysis was performed on mRNAs extracted from independent preparations of primary hepatocytes (3 mice for each condition). More than 70% of the genes analyzed were equally regulated in both the original microarray data and the PCR TLDAs, thus validating the array screening. We used the

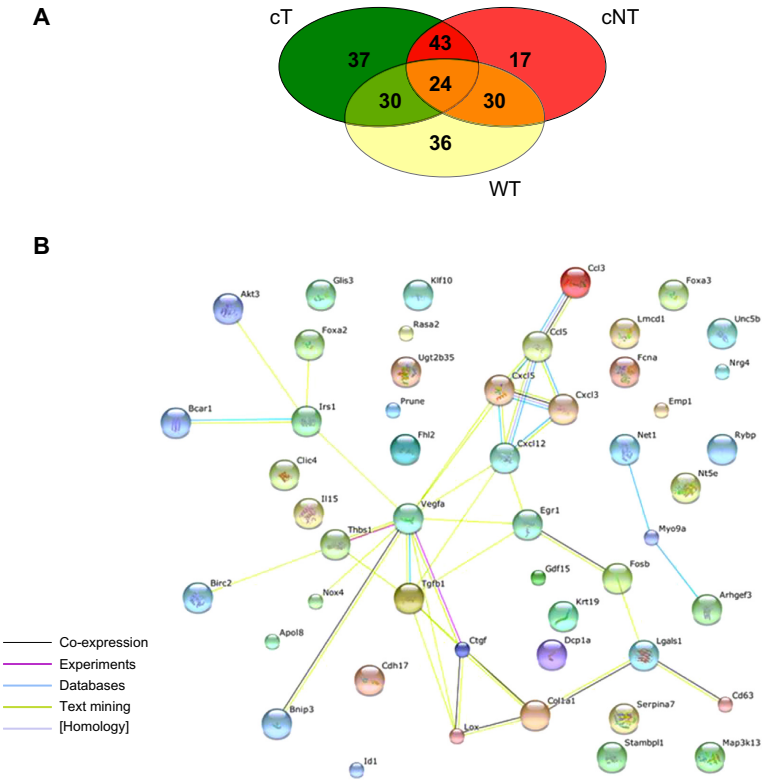

Fig. 1. Gene expression profile of TGF-β1-treated mouse hepatocytes expressing HCV core. (A) Venn diagram representation of differential gene expression in control and core-expressing primary hepatocytes. (B) String network analysis of genes modulated by core and validated in TLDA. The color pattern depicting the associations is explained on the left-hand side.

String 9.0 pathway analysis tool to examine the network of protein interactions between the gene products included in our signature. Interestingly, a network connecting several TGF-β1 target genes could be identified as specifically induced by HCV core (Fig. 1B). TGF-β1 was placed in center network association and especially in direct relation to genes related to fibrosis such as *CTGF*, *LOX*, *THBS1*, *VEGF*, *EGR1*, and *COL1A1*; description and score quantification of these interconnectivities are shown in the Supplementary Table network analysis. GO term enrichment analyses suggested that the genes, upregulated by both cT and cNT core proteins, are related to those biological processes involved in cancer development, and positive regulation of cytoskeleton while the downregulated genes are related to chemokine expression (Supplementary Material). Moreover, none of the TGF-β1 target genes that mediate growth inhibition such as *p21* or *p16* were induced by HCV core proteins; a finding in accordance with our previous observations that HCV core attenuates the cytostatic effects of TGF-β1. Importantly, comparison of gene expression profiles in the TGF-β1 treated or untreated cT and cNT- expressing hepatocytes showed the same expression changes in TGF-β1 treated and non-treated cells. This suggests that HCV core is able to induce TGF-β1 target gene expression in the absence of exogenous TGF-β1 stimulation. These TGF-β1 target genes represent more than 50% of the genes modulated by HCV core expression (Table 1). Although both cNT and cT were capable of activating TGF-β1 target genes, some genes, such as *Id1*, *IL-15*, or *Net1* were differentially modulated in cells expressing cNT or cT. This observation supports our previous findings showing that core proteins derived from tumor and non-tumor liver differently respond to TGF-β and might exert different biological effects [14].

HCV core activates latent TGF-β

The ability of HCV core to upregulate TGF-β1 target gene expression in primary hepatocytes from transgenic mice in the absence of exogenous TGF-β1 stimulation might be explained by a core-dependent increase in active TGF-β levels. To substantiate this hypothesis, we determined the levels of the active form of TGF-β1 in liver extracts from HCV core transgenic mice and found that these extracts exhibit statistically higher levels of active TGF-β than their control littermates (Fig. 2A). When total TGF-β levels of liver extracts of control and transgenic mice were compared, no statistical difference could be found (Supplementary Fig. 2A).

The ability of HCV core to increase active TGF-β levels is also supported by experiments performed in HuH7 cells. We first determined whether HCV core expressing cells could activate a TGF-β transcriptional response. HCV core binds to Smad3 and inhibits Smad3 transcriptional responses but does not bind Smad2 and does not affect Smad2 transcriptional activity [21,13]. To determine TGF-β transcriptional response, HuH7 cells were transiently transfected with either cNT or cT core vectors together with the Smad2 responsive ARE-luc reporter plasmid and with FoxH1 expression vector. As shown in Fig. 2B, luciferase activity was higher in HuH7 cells expressing either cNT or cT as compared to control HuH7 cells showing that both cores were capable of activating TGF-β transcriptional responses. Luciferase activity was totally reversed by treating the cells with SB431542, an inhibitor of TGF-β1 receptor, thus suggesting that the activation of TGF-β signaling was indeed due to the binding of active TGF-β to its specific receptor.

The ability of HCV core to activate TGF-β signaling was a general property of HCV core since a collection of previously described HCV core isolates from tumor or non-tumor liver tissues [22] was able to activate TGF-β signaling, irrespective of their origin (Supplementary Fig. 3).

We then established HuH7 cells stably expressing HCV cNT or cT and determined Smad2 phosphorylation as well as active TGF-β levels. We first verified that Smad2-dependent transcriptional activity was increased in cNT or cT stably expressing cells as compared to controls in a manner comparable to transient transfected cells (Supplementary Fig. 4).

Next we determined Smad2 phosphorylation, which reflects the engagement of TGF-β signaling, in HuH7 cells stably expressing either cNT or cT proteins. Core expression in these cells is shown in Fig. 2C. Smad2 phosphorylation was increased in HuH7 cells expressing either cNT or cT when compared to control cells and this phosphorylation was completely abolished when cells were treated with SB431542 (Fig. 2C). The same effect was observed in HuH7.5 cells stably expressing the full-length HCV replicon (Fig. 2C). To verify whether this increased TGF-β signaling was also observed in more physiological conditions, we studied Smad2 phosphorylation in HuH7.5 cells infected for 72 h with

Table 1. List of genes specifically regulated by HCV core T or NT validated by PCR TLDAs.

Upregulated genes			Downregulated genes		
cT	cNT	Both	cT	cNT	Both
BCAR1	*APOL8*	*BNIP3*	*BIRC2**	*AKT3*	*CCL5**
*COL1A**	*CD63*	*CXCL12**	*CXCL5**	*CDH17**	*CXCL3**
*CTGF**	*IL15**	*LGALS1**	*DCP1A*	*ID1**	*FCNA*
*EMP1**	*LMCD1**	*NET1**	*EGR1*		*LOX*
KRT19	*RASA2**	*NOX4**	*FHL2*		*MYO9A**
*PRUNE**			*FOXA3*		*NRG4**
*RYBP**			*GAS5*		*SERPINA7*
STAMPLI1			*KLF10**		
STMN1			*MAP3K13*		
*THBS1**					

*TGF-β1 target genes.

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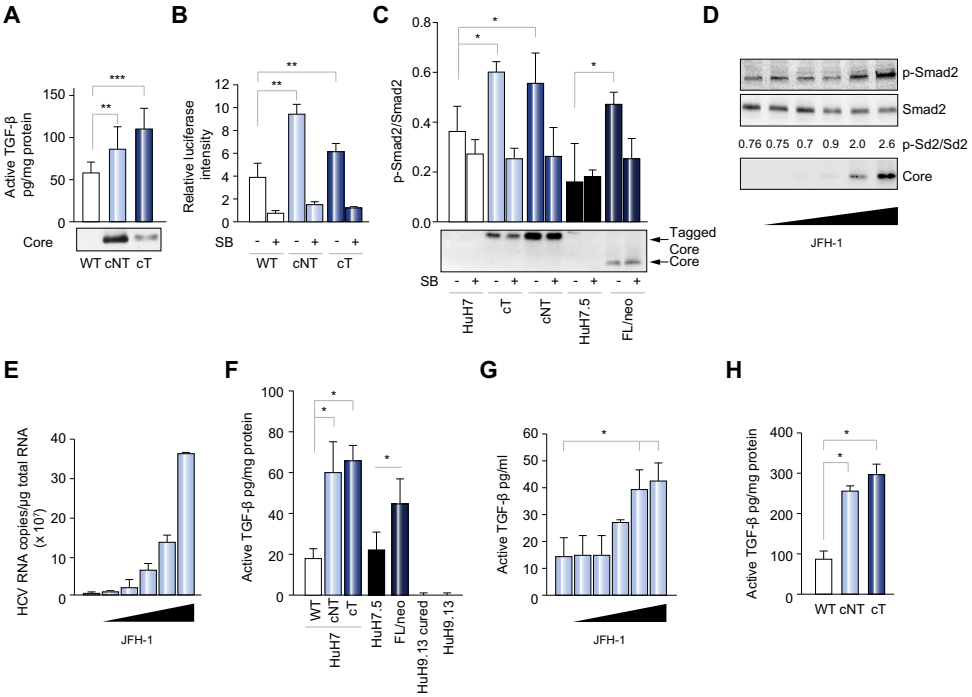

Fig. 2. HCV core induced TGF-β signaling in HuH7 cells. (A) Active TGF-β levels were determined in liver extracts from mice transgenic for HCV cores or their controls (WT). Results represent the mean ± SD of TGF-β1 levels in five 9-month-old males/condition. Core expression in liver extracts of one representative mouse WT, transgenic for cNT or cT core is shown. (B) Smad2 transcriptional activity in HuH7 cells transiently transfected with *ARE*-luc and FoxH1 plasmids together with vectors coding for cNT or cT and treated or not with SB431542 (1 μM) (SB). Luciferase activity was measured 24 h after transfection. Results represent the mean ± SD of 3 independent experiments performed in triplicates. (C) HuH7 cells expressing cNT, cT or the full-length replicon (FL/neo), and their respective controls were cultured for 48 h with or without SB431542. Protein extracts were analyzed for p-Smad2 expression by Western blotting. Densitometric analysis of p-Smad2/Smad2 ratios is shown as the mean ± SD of 3 independent experiments. (D) HuH7.5 cells were infected with increasing concentrations of JFH1 (0.5, 1, 5, 20, and 50 HCV mRNA copies/cell). Protein extracts were analyzed for p-Smad2, Smad2 and core expression by Western blotting. (E) HCV RNA copies were determined by qPCR 72 h after JFH1 infection of HuH7.5 cells. (F) HuH7 cells expressing cNT, cT, the full length replicon (FL/neo), or the subgenomic replicon (HuH9.13) and their respective controls were incubated for 48 h without serum before determination of active TGF-β levels in cell culture supernatants. Results represent the mean ± SD of 3 independent experiments performed in triplicates. (G) Active TGF-β levels were determined in culture supernatants of HuH7.5 cells infected with various concentrations of JFH1 for 72 h. (H) HuH7 cells expressing or not HCV cores were incubated for 48 h without serum in presence of latent TGF-β1 (50 ng/ml) before determination of active TGF-β1 levels. Results represent the mean ± SD of 3 independent experiments performed in triplicates. *p <0.05, **p <0.01, ***p <0.001.

different concentrations of JFH1. In these experimental conditions, Smad2 phosphorylation was increased in a dose-response manner (Fig. 2D), which was correlated with HCV copies (Fig. 2E).

To assess whether this Smad2 phosphorylation and transcriptional activation resulted from an increased activation of TGF-β, active TGF-β levels were determined in the supernatants of cells grown in serum-free medium. Active TGF-β was barely detectable in the supernatants from control HuH7 cells while it was consistently found, and statistically different from controls in the supernatant of HCV core expressing cells as well as in the supernatant of cells stably expressing the full length HCV replicon. (Fig. 2F). The same increase in active TGF-β was observed in HuH7.5 cells after infection with JFH1 (Fig. 2G).

By contrast, active TGF-β was not increased in HuH9.13 cells expressing the NS3-NS5B subgenomic replicon when compared to HuH9.13-cured cells (Fig. 2F) and here again was dependent on JFH1 concentrations. Total TGF-β levels were comparable in all the cell lines (Supplementary Fig. 2B). These observations indicate that expression of HCV core in HuH7 was able to activate endogenous TGF-β. Moreover, when HuH7 cells were incubated with exogenously added latent TGF-β1, active TGF-β levels were significantly higher in supernatants of cells expressing HCV cores than in supernatants of control cells (Fig. 2H).

Altogether, these results demonstrate that HCV core initiates TGF-β signaling by promoting the conversion of latent TGF-β1 into the active form.

Active TGF-β1 induced by HCV core mediates stellate cell activation

Chronic HCV infection progression is strongly correlated with the accumulation of liver fibrosis. Since hepatic stellate cells (HSCs), the main source of the excess ECM produced in liver fibrosis,

are activated *in vivo* in chronic hepatitis C patients [23], we sought to determine whether TGF-β1 activation induced by HCV core expression in infected hepatocytes could exert a paracrine effect on these non-parenchymal liver cells. As shown in Fig. 3A, exposure to a conditioned medium from HCV core expressing HuH7 cells was able to activate TGF-β signaling (as shown by the increase in pSmad2 levels) in the stellate cell line LX-2, and this activation was abolished by SB431542. To confirm this result, LX-2 cells transfected with a Smad3-responsive luciferase reporter plasmid were incubated as above with the conditioned medium from control or HCV core expressing HuH7 cells. Here again, luciferase activity was significantly increased by HCV core conditioned medium and this effect was abrogated by SB431542 treatment (Fig. 3B).

These results strongly suggest that the TGF-β1 activation induced by HCV core in hepatocytes signals to both hepatocytes and neighboring cells. This notion is further supported by the strong increase in αSMA polymerization, a marker of HSCs activation, observed by immunofluorescence staining in LX-2 cells co-cultured with HuH7 cells expressing HCV cNT or cT (Fig. 3C). As control, a clear polymerization of αSMA was present in LX-2 cells treated with TGF-β1 whereas αSMA was never detected in HuH7 cells (Fig. 3C). This result was confirmed by Western blot analysis where basal expression of αSMA in LX-2 cells was increased by culture with conditioned medium from HuH7 cells expressing HCV core as compared to conditioned medium from HuH7 control cells and this effect was reversed in the presence of SB431542 (Fig. 3D). This result strongly suggests that TGF-β released in the culture medium is responsible for αSMA expression.

HCV core-induced TSP-1 mediates TGF-β activation

Since HCV core expressing cells were capable of activating exogenously added latent TGF-β1, we speculated that the activation might occur in the secreted form of TGF-β. The extracellular matrix protein TSP-1 has been shown to bind to the Leu-Ser-Lys-Leu (LSKL) amino acids sequence at the amino terminus of LAP in the TGF-β latent complex, to alter its conformation and to render TGF-β biologically active [24]. LSKL peptide functions as a competitive antagonist of TSP-1 binding to the latent complex and inhibits TSP-1-dependent TGF-β activation both *in vitro* and *in vivo* [25].

TSP-1 was upregulated in HCV core expressing cells in our gene profiling analyses, suggesting that increased TSP-1 expression could contribute to the enhanced TGF-β activation. We first determined whether *TSP-1* mRNA upregulation observed in mouse hepatocytes was also observed in HuH7 cells expressing HCV core and was translated into higher TSP-1 protein expression. As shown in Fig. 4A, *TSP-1* mRNA levels were significantly higher in HuH7 cells expressing cT core than in control cells. Accordingly, protein levels were increased in cell culture supernatants from HuH7 cells stably expressing HCV core and primary hepatocytes isolated from HCV cNT and cT transgenic mice, as compared to their control littermates (Fig. 4B). To examine whether the LSKL peptide was able to decrease TGF-β1 activation in HCV core expressing cells, confluent cells were incubated with LSKL peptide in the presence or not of latent TGF-β1 in serum free medium and active TGF-β1 was determined in the supernatants. As expected, active TGF-β was increased in HCV core expressing cells when compared to controls. LSKL reduced active TGF-β levels and this effect was more pronounced when the cells were

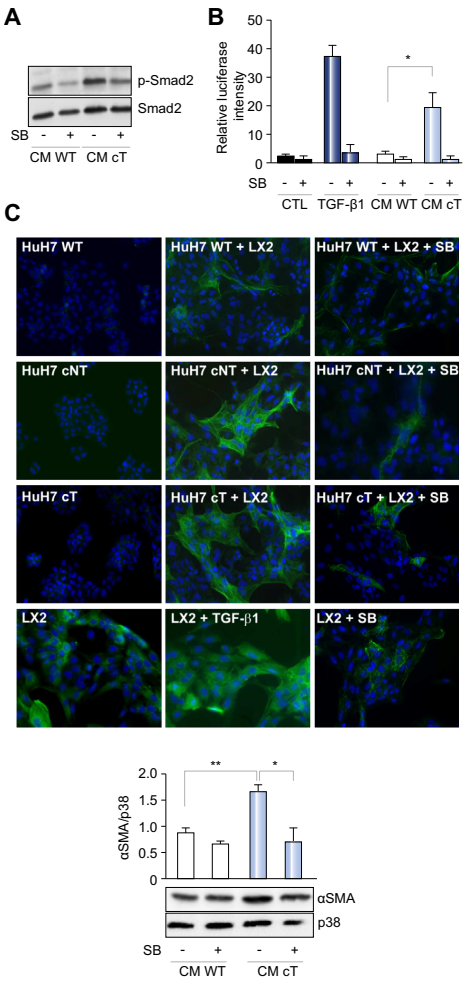

Fig. 3. HCV core proteins activate hepatic stellate cells through TGF-β signaling. (A) LX-2 cells were cultured for 24 h with conditioned medium (CM) from HuH7 cells expressing or not cT in the presence or not of SB431542 (1 μM). Protein extracts were analyzed for p-Smad2 expression. One out of 3 representative experiments is shown. (B) LX-2 cells were transfected with the *SBE*-luc reporter plasmid and cultured for 24 h with or without CM from HuH7 control or core expressing cells. Smad3 transcriptional activity was determined 24 h later by luciferase activity. Results represent the mean ± SD of 3 independent experiments performed in triplicates. (C) LX-2 cells were co-cultured with HuH7 cells expressing or not HCV cNT or cT for 48 h (1/1 ratio) and αSMA expression was determined by immunofluorescence analysis. (D) LX-2 cells were cultured for 48 h with conditioned medium (CM) obtained from HuH7 cells expressing or not cT in the presence or not of SB431542 (1 μM). Protein extracts were analyzed for αSMA expression. Densitometric analyses of αSMA/p38 ratio represent the mean ± SD of 3 independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.01.

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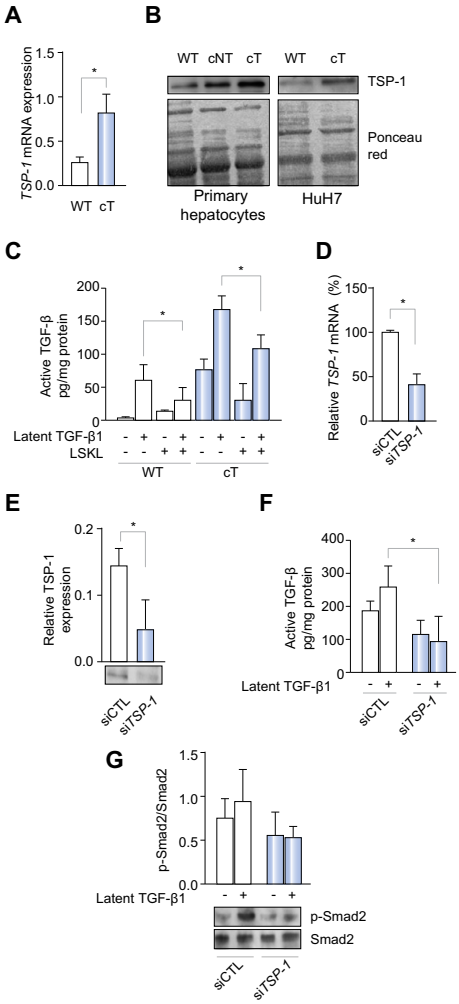
incubated with latent TGF-β1 (Fig. 4C). Next, HCV *c*T expressing HuH7 cells treated or not with latent TGF-β1 were transfected with *TSP-1* siRNA. *TSP-1* mRNA expression and secreted TSP-1 protein were both significantly decreased by *TSP-1* siRNA transfection (Fig. 4D and E). *TSP-1* silencing inhibited both the activation of latent TGF-β1 (Fig. 4F) and Smad2 phosphorylation (Fig. 4G) in HCV core expressing cells, strongly suggesting that TSP-1 contributes to core-mediated TGF-β1 activation.



Discussion

In this report, we provide evidence that HCV core can provoke TGF-β activation through an increased secretion of TSP-1 and induce a profibrotic response of hepatic stellate cells. Extensive studies have reported an increase in TGF-β1 levels in patients chronically infected with HCV. However, until now no study has reported whether the active form of TGF-β was correlated with levels of total TGF-β. High levels of latent TGF-β are present in different organs and TGF-β responses are restricted by availability of active TGF-β that occurs locally and transiently in response to different stimuli. TGF-β activation is thus considered as a major step in TGF-β responses.

In vitro experiments demonstrated that HCV infected hepatocytes, when co-cultured with CD4 lymphocytes, were capable of directly inducing Tregs development through production of TGF-β, suggesting that HCV not only induced TGF-β expression, but could also be able to activate the cytokine [26]. Moreover, in an HuH7.5-FL HCV replicon system and in HepG2 cells transfected with HCV JFH1 RNA, it has been demonstrated that HCV stimulated CTGF through TGF-β1 driven production of key fibrosis-associated biomarkers [27]. Our findings, by showing TGF-β1 is activated in HCV JFH1-infected cells as well as in HuH7 cells expressing all HCV proteins, support this hypothesis. We did not observe such results in cells expressing only the HCV non-structural proteins, hence suggesting that the core protein expression was sufficient to provoke TGF-β1 activation. Indeed, we could detect active TGF-β1 in the supernatant of HuH7 cells stably, only expressing HCV core. Smad2 phosphorylation and Smad2 transcriptional activity were increased in these cells and these effects were totally abolished in the presence of a TGF-β1 receptor inhibitor.

Our microarray analysis demonstrated that HCV core expression in hepatocytes isolated from transgenic mice modulates TGF-β1 target gene expression. Interestingly, only a subset of TGF-β1 target genes is induced by HCV core when compared to hepatocytes from control mice treated with TGF-β1 where a lar-

Fig. 4. HCV core-mediated TGF-β activation through TSP-1 expression. (A) *TSP-1* mRNA levels from HuH7 cells expressing or not core protein were determined by qRT-PCR. Results are expressed as relative *TSP-1* mRNA levels normalized to *RPLP0* mRNA taken as housekeeping gene and represent the mean of three independent experiments. (B) Cells were incubated for 48 h in serum free medium, culture supernatants were concentrated and equal amounts of proteins were analyzed by Western blotting using an anti-TSP-1 antibody. In the absence of a specific protein loading control for secreted proteins, Ponceau red staining of the blots is shown. (C) HuH7 cells expressing or not HCV core were incubated for 48 h with or without latent TGF-β1 (50 ng/ml) or a TSP-1 inhibitor LSKL (5 μM). Active TGF-β levels were determined in culture supernatants. Results represent the mean ± SD of 3 independent experiments performed in triplicates. (D) HuH7 cells expressing core protein were transfected with *TSP-1* siRNA and *TSP-1* mRNA levels were determined by qRT-PCR 96 h later. The graph represents the mean of three independent experiments. (E) TSP-1 protein expression in the supernatants of control and siRNA transfected cells. A representative blot of TSP-1 and densitometric analysis of 3 independent experiments are shown. (F and G) HuH7 cells expressing core protein were transfected with *TSP-1* siRNA and treated or not 48 h later with latent TGF-β1 for another 48 h before determination of active TGF-β levels (F) or Smad2 phosphorylation. A representative blot and densitometric analysis of 3 independent experiments are shown (G). *p <0.05.

ger panel of genes is regulated. It is known that differences in the binding affinity of Smad3 on target gene promoters determine the nuclear concentration of a transcriptional activator that is required for a gene to be switched on. Thus the lower (bioactive) TGF-β concentrations in the supernatant of HCV core expressing hepatocytes compared with externally added bioactive TGF-β1 in control hepatocytes might account for expression of a specific subset of TGF-β-target genes. Since we have already observed that HCV core alleviates Smad3 dependent transcription, it is also conceivable that expression of only some TGF-β-target genes in core expressing cells is a reflection of this inhibition. It is possible that HCV core protein is able to activate alternate pathways to modify expression of transcription factors or cofactors that interfere with Smad to drive TGF-β target gene expression. Moreover, none of the genes involved in growth arrest by TGF-β1 via interference with cell cycle progression (such as p15 or p21) were found to be modulated by core. GO term enrichment analyses suggested that genes upregulated by both tumor- and non-tumor-derived cores were related to fibrosis development. Furthermore, although tumor- and non-tumor-derived cores activate TGF-β1 in comparable amounts, they drive a different pattern of TGF-β target gene expression. These observations are consistent with our previous work, which demonstrated that tumor- and non-tumor-derived cores did not bind to Smad3 with the same affinity and did not activate Smad3 responsive gene in the same way, even though the same amounts of TGF-β were applied to these cells [14]. Thus these overall observations offer a novel paradigm whereby, with the same level of TGF-β activation, different TGF-β target gene expression is driven by tumor- and non-tumor-derived cores.

Our gene profiling analyses have identified *THBS1* as a gene upregulated in core expressing mouse hepatocytes. This gene encodes for thrombospondin-1, a secreted protein implicated in TGF-β1 activation. Our data using an inhibitor of TSP-1 binding to latent TGF-β1 or *TSP-1* silencing by siRNA are in favor of a role for this protein in core mediated TGF-β1 activation. A recent study demonstrated that TGF-β1 could be activated in HCV infected cells through the protease activity of furin or TSP-1 before secretion [28]. In contrast with this result and in agreement with the increasing evidence that TGF-β1 activation occurs in the extracellular space, we here show that TGF-β1 is secreted in its latent form and activated later. Moreover, our results demonstrate that core is the protein responsible for TGF-β activation in HCV infection.

Whatever the mechanisms by which HCV core increases the bioactivity of TGF-β, the data accumulated in the present study demonstrate that this increased bioactivity of TGF-β was capable of promoting TGF-β1 signaling and αSMA expression in hepatic stellate cells co-cultured with HCV core expressing cells. Thus it is conceivable that, *in vivo*, the interaction of HCV infected hepatocytes with hepatic stellate cells could result in hyperactivity of TGF-β signaling and subsequent differentiation of these hepatic stellate cells.

Activation of stellate cells by HCV core expressing cells has already been described. It was reported that increased expression of TGF-β1 HuH7 cells expressing HCV replicon was responsible for HSCs profibrotic effects [29]. Another report implicated IL-8 as the major cytokine involved in this activation [30]. In lung fibroblasts, it was shown that TGF-β stimulates IL-8 release. So, it would be of interest to determine IL-8 levels in co-culture of LX-2 cells and HCV core expressing HuH7 cells to establish if TGF-β-mediated modulation of IL-8 could contribute to LX-2 activation. Of note, the connection between TGF-β1 activation through TSP-1 in HCV infected cells and activation of hepatic stellate cells has been reported recently [31], further supporting our present findings.

We have previously reported that HCV core variants were capable of shifting TGF-β1 responses from tumor suppressor effects to tumor promotion by decreasing hepatocyte apoptosis and increasing EMT. This effect was mainly attributed to the capacity of HCV core to alleviate Smad3 signaling. Our findings provide additional insights into HCV core interactions with the TGF-β pathways and bring to light a dual role of a viral protein in liver fibrosis and HCC. Thus, HCV core modulates TGF-β1 responses in HCV-infected hepatocytes and affects liver stromal cells in a paracrine fashion through TGF-β activation. Taken together, our data suggest that selective targeting of excessive TGF-β activity through blockade of TSP-1-dependent TGF-β activation could represent a therapeutic strategy for treating HCV-induced liver pathologies.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2013.07.036.

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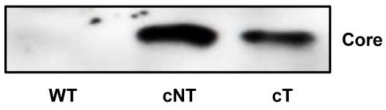
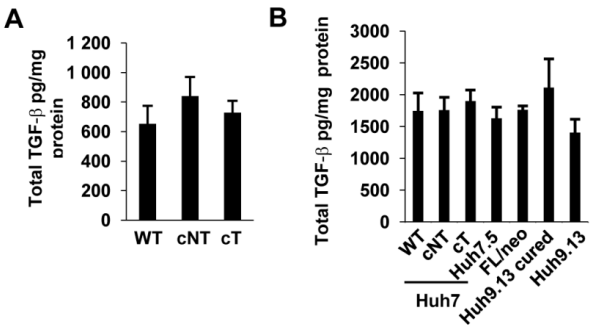
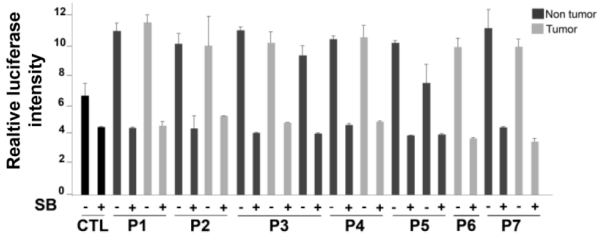


Supplementary Fig. 1. Hepatocytes isolated from 2 month old mice transgenic for either cNT or cT core or their control littermates were cultured for 24h and core expression was evaluated by western blot analysis.

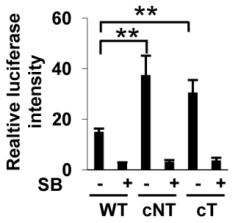


Supplementary Fig. 2. A. Total TGF-b levels in liver extracts from WT or mice transgenic for HCV cNT or cT cores.
B. Total TGF-b levels in cell culture supernatants of Huh7 cells stably expressing HCV cNT, cT, the full-length replicon (FL/neo) or the NS3-NS5A subgenomic replicon (Huh9.13).



Supplementary Fig.3. Smad2 transcriptional activity of Huh7 cells transiently transfected with ARE-luc and FoxH1 plasmids together with vectors coding for different variants of HCV core isolated from tumor or non tumor (from 7 patients) and treated or not with SB431542.

Luciferase activity was measured 24h after transfection.



Supplementary Fig. 4. Huh7 cells control or stably expressing HCV cores were transfected with an ARE-luc reporter plasmid together with FoxH1 and luciferase activity was measured 24h later.

Results represent the mean +/- SD of 3 independent experiments realized in triplicates

Supplementary Material

Materials and methods

Vectors

HCV core sequences isolated from c1b genotype have been previously described (Battaglia et al., PLoS One. 2009;4(2):e4355). The T sequence differs from the NT one by 2 changes in aa 118 (N/D) and aa 189 (A/V). They were cloned in the bicistronic expression vector pBICEP-CMV2-3xFlag (Invitrogen). Stable transfectants were obtained by selection with G418 (400μg/ml) for 3 weeks and a pool of resistant cells was used to avoid differences due to transgene insertion. The luciferase reporter plasmids FoxH1 and (ARE)3-luc were used to determine Smad2 transcriptional activity and (SBE)9-luc to determine Smad3 transcriptional activity as previously described (Seo et al., Mol. Cell 2006 Aug;23(4):547-59)

Microarrays

Total RNA was extracted from samples by Trizol (Invitrogen, Carlsbad, CA, USA), purified from genomic DNA contamination through a DNase I (Qiagen, Valencia,CA, USA) digestion step and further enriched by Qiagen RNeasy columns for gene expression profiling (Qiagen). Quantity and integrity of the extracted RNA have been assessed by NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Expression profiles have been determined by using the Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, the Affymetrix GeneChip® Whole Transcript Sense Target Labelling Assay has been used to amplify and reverse-transcribed total RNA (300 ng), and to biotinylate sense-strand DNA targets. Arrays has been hybridized with the labeled-target hybridization cocktail by rotation at 60 rpm in the Affymetrix Gene Chip hybridization oven at 45 °C for 16 h, washed in the Affymetrix GeneChip Fluidics station FS 450, and scanned by Affymetrix Gene Chip scanner 3000 7G system, to produce image files (.CEL).

Normalized intensities were transformed into log2 ratio to be analysed by TM4

MultiExperimental Viewer software (TMEV version 2.0). The transcriptome of TGF-β1 treated cells was compared to that of their respective controls by Significance Analysis of Microarray (SAM) algorithm with a fold change >2 and False Discovery Rate (FDR) <1. This allowed us to build a Venn diagram and to determine a TGF-β1 expression profile for each condition. The microarray data of each gene list were loaded in TMEV2.0 software to verify the prediction class of microarray experimental groups by using an unsupervised ascending hierarchical clustering algorithm. Gene ontology classification of Biological Process and information concerning pathway (KEGG and Biocarta) on HCV core gene list (verified by TLDA) was extracted from website NIH DAVID Bioinformatics application (http://david.abcc.ncifcrf.gov/. Molecular Network based on interaction and data-mining was realized on HCV core gene list (verified by TLDA) using website application STRING 9.0 (http://string-db.org/).

Taqman® Low Density Arrays (TLDAs)

Total RNA was extracted from cells with the Qiagen RNAeasy kit according to manufacturer's instructions (Qiagen, Valencia, CA, USA) and retro-transcribed with the Invitrogen (Carlsbad, CA, USA) Superscript II kit using random examers as template. Quantity and integrity of the extracted RNA and synthetized cDNA have been assessed by NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). 200ng of cDNA were loaded in double on the Low Density Arrays (Applied Bioystems, Foster City, CA, USA) customized with 95 probes directed to 95 target genes and 18s probe as a control. The TLDAs were run on an AB 7900HT machine, real time PCR data were collected and analyzed through the SDS 2.2 program (Applied Biosystems, Foster City, CA, USA).

Quantitative RT-PCR

Total cell RNA was isolated using RNeasy kit (Qiagen) or RNAble, (Eurobio). The concentration of total RNA was determined using a Nanodrop Spectrophotometer ND-1000 (Fisher Scientific SAS, Illkirch, France).

For TSP-1 mRNA determination, qRT-PCR was performed in duplicate using TSP-1-designed primers from Origen. RNA was extracted with an RNeasy kit (Qiagen). RT was realized with a Revert Aid Premium First Strand (Fermentas) and qPCR with Light Cycler Fast Start DNA master SyBR Green1 (Roche Diagnostics).

The ribosomal protein large P0 (RPLP0) mRNA was used for the normalization of expression data. siRNA duplexes targeting TSP-1 [sequence CCACGAGGGCUCAGGGAUA] were from Dharmacon.

HCV RNA copy number was quantitated using the SuperScript III Platinium One-Step quantitative RT-PCR system (Invitrogen).

Conditioned medium

Huh7 cells were cultured to confluence with complete DMEM medium containing 10% FCS. They were then washed 3 times in serum free medium and cultured in this medium for 48 h. Cell culture supernatants were centrifuged to eliminate cell debris and stored at -80°C. For western blot analysis, these conditioned media were concentrated about 50 times on Amicon Ultracell 10K centrifugal filters (Millipore) and protein concentration was estimated by Bradford method.

Antibodies

Antibodies directed against HCV core protein (Abcam), phospho-Smad2 (Cell signaling), Smad2 (Zymed), Flag tag (M2 Sigma) TSP-1 (Clone A6.1 Labvision) or αSMA (Clone 1A4 Sigma) were used.

Determination of active TGF-β levels

The assay based on a biological activity of TGF-β that thus detects only the active form of the cytokine was previously described (Rogier et al Kidney Intl. 2005 Oct;68(4):1875-83). Briefly, cells stably transfected with a reporter plasmid expressing the luciferase gene downstream of Smad3 binding sequences (SBE)-9, express luciferase activity in response to active TGF-β in a dose dependent manner. Samples were incubated in the presence of these cells for 18h. Relative luciferase activity was determined using a luciferase assay system kit from Promega according to the manufactory instructions. Acid pH can activate latent TGF-β. So, to estimate latent TGF-β levels, samples were acidified with HCL for 10 min RT and pH was neutralized with NaOH/Hepes before adding the samples to the cells. TGF-β concentrations were calculated from the luciferase activity using a standard curve obtained for each experiment with known amounts of TGF-β.

Livers obtained from 9 month-old mice transgenic or not for either T or NT core were homogenized in disposable tissue grinders in PBS supplemented with a cocktail of antiproteases and antiphosphatases. After centrifugation, supernatants were used for TGF-β levels determination.

Legends to Tables

String network analysis

Network description of Gene regulated by the HCV core interactions performed with STRING 9.05 on website (http://string-db.org). Row description from the left to the right: node1 and node2: names of genes implicated in the node: score of protein homology; coexpression: score of coexpression in *Mus musculus*, experiments: score based on experiments such as coimmunoprecipitation, complex assay, in vivo assay, affinity capture-western assay, purification assay etc.… knowledge: score from curated database; testmining : score of co-citation in PubMed abstract and finally combined score: combination of all previous scores. (Scale of scores: low confidence: 0.150; medium confidence: 0.400; high confidence: 0.700; highest confidence: 0.900)

Supplementary GO annotation tables

The annotation of Gene Ontology was performed by using INCROMAP cross plateform microarray software, which can access to GO databases by parsed pipeline web communication.

Gene ontology annotation were separated in 3 terms: BP: biological process, MF: molecular function, CC: cellular components. So we studied the 3 terms of GO annotation concerning genes up or down regulated by core. In each annotation, when possible, we kept the best 50 possibilities.

BP up core

Rank	Name	List ratio	BG ratio	P-value
1	AGING	16//35	13/75605	3.705579181762938E-60
2	S_PHASE_OF_MITOTIC_CELL_CYCLE	14//35	10/75605	1.7633167949894427E-54
3	S_PHASE	15//35	14/75605	1.8813825584708468E-53
4	RESPONSE_TO_STEROID_HORMONE_STIMULUS	14//35	11/75605	7.756438588798729E-53
5	EMBRYONIC_DEVELOPMENT	19//35	58/75605	9.44149694356094E-52
6	B_CELL_DIFFERENTIATION	14//35	12/75605	2.7915421866468666E-51
7	MORPHOGENESIS_OF_AN_EPITHELIUM	15//35	16/75605	4.5129290993205283E-51
8	REGULATION_OF_MITOTIC_CELL_CYCLE	16//35	23/75605	1.826834166476775-50
9	INTERPHASE	19//35	68/75605	3.333400101273272665-50
10	CELL_FATE_COMMITMENT	14//35	13/75605	7.255993388343066E-50
11	ACTIVATION_OF_NF_KAPPAB_TRANSCRIPTION_FACTOR	15//35	18/75605	2.3003760416244807E-49
12	POSITIVE_REGULATION_OF_CYTOSKELETON_ORGANIZATION_AND_BIOGENESIS	13//35	10/75605	3.3920957239746375E-49
13	POSITIVE_REGULATION_OF_DNA_BINDING	16//35	26/75605	3.955156380367061 4E-49
14	CELL_CYCLE_ARREST_GO_0007050	18//35	57/75605	1.3744866136690222E-48
15	PROTEIN_UBIQUITINATION	17//35	40/75605	1.6558345508966694E-48
16	MEMBRANE_FUSION	16//35	28/75605	2.2640871404337257E-48
17	REGULATION_OF_CELL_MIGRATION	16//35	28/75605	2.2640871404337257E-48
18	POSITIVE_REGULATION_OF_BINDING	16//35	28/75605	2.2640871404337257E-48
19	PROTEIN_MODIFICATION_BY_SMALL_PROTEIN_CONJUGATION	17//35	43/75605	7.853867129449438E-48
20	INTERPHASE_OF_MITOTIC_CELL_CYCLE	18//35	62/75605	8.180697349739707E-48
21	RAS_PROTEIN_SIGNAL_TRANSDUCTION	18//35	66/75605	3.027385448685357 3E-47
22	EXTRACELLULAR_STRUCTURE_ORGANIZATION_AND_BIOGENESIS	16//35	32/75605	4.468941241339662E-47
23	REGULATION_OF_DNA_BINDING	17//35	47/75605	5.106807672351522E-47
24	JNK_CASCADE	17//35	47/75605	5.106807672351522E-47
25	UBIQUITIN_CYCLE	17//35	48/75605	7.905431364324002E-47
26	STRESS_ACTIVATED_PROTEIN_KINASE_SIGNALING_PATHWAY	17//35	49/75605	1.2102307946243736E-46
27	POSITIVE_REGULATION_OF_CELL_CYCLE	14//35	16/75605	1.2179912108347452E-46
28	POSITIVE_REGULATION_OF_TRANSCRIPTION_FACTOR_ACTIVITY	15//35	24/75605	3.680121136180581E-46
29	EMBRYONIC_MORPHOGENESIS	14//35	17/75605	6.900032707935518E-46
30	NEGATIVE_REGULATION_OF_CELL_CYCLE	18//35	79/75605	1.2132897681001149E-45
31	MITOSIS	18//35	82/75605	2.577198559616262 7E-45
32	NEGATIVE_REGULATION_OF_ANGIOGENESIS	13//35	13/75605	3.4894533931136E-45
33	GLAND_DEVELOPMENT	13//35	13/75605	3.4894533931136E-45
34	REGULATION_OF_BINDING	17//35	58/75605	3.67067900420674E-45
35	REGULATION_OF_TRANSCRIPTION_FACTOR_ACTIVITY	16//35	40/75605	4.663567232174353E-45
36	M_PHASE_OF_MITOTIC_CELL_CYCLE	18//35	85/75605	5.310052039158 91E-45
37	ACTIVATION_OF_MAPK_ACTIVITY	16//35	41/75605	7.646327191357062E-45
38	ACTIVATION_OF_PROTEIN_KINASE_ACTIVITY	15//35	28/75605	1.052737822585747 1E-44
39	CELL_CYCLE_PHASE	20//35	170/75605	1.078043067805743 4E-44
40	T_CELL_PROLIFERATION	14//35	19/75605	1.179250072773 99E-44
41	SMALL_GTPASE_MEDIATED_SIGNAL_TRANSDUCTION	18//35	89/75605	1.333164273770479 6E-44
42	BONE_REMODELING	15//35	29/75605	2.180094131803333 2E-44
43	B_CELL_ACTIVATION	14//35	20/75605	3.929741474437908E-44
44	REGULATION_OF_JNK_ACTIVITY	14//35	20/75605	3.929741474437908E-44
45	POSITIVE_REGULATION_OF_ANGIOGENESIS	12//35	10/75605	4.346818763901760 6E-44
46	EPITHELIAL_TO_MESENCHYMAL_TRANSITION	12//35	10/75605	4.346818763901760 6E-44
47	REGULATION_OF_ENDOTHELIAL_CELL_PROLIFERATION	12//35	10/75605	4.346818763901760 6E-44
48	TISSUE_REMODELING	15//35	30/75605	4.359034407488614E-44
49	REGULATION_OF_CELL_CYCLE	20//35	182/75605	4.556541277784958E-44
50	TISSUE_MORPHOGENESIS	13//35	14/75605	4.883812972391 17E-44

BP down core

Rank	Name	List ratio	BG ratio	P-value	Genes
1	RHO_PROTEIN_SIGNAL_TRANSDUCTION	06//14	38/75605	3.185782253833126E-17	CCL5, CXCL5,NET1 , GDF15 , BIRC2 , IRS1
2	REGULATION_OF_JNK_CASCADE	05//14	12/75605	7.696856822412638E-17	CCL5,NET1 , GDF15 , BIRC2 , IRS1
3	RAS_PROTEIN_SIGNAL_TRANSDUCTION	06//14	66/75605	1.045391699713 5432E-15	CCL5 ,CXCL5 ,NET1 ,GDF15 ,BIRC2 , IRS1
4	REGULATION_OF_MAPKKK_CASCADE	05//14	20/75605	1.505283461483 1433E-15	CCL5 , NET1 ,GDF15 , BIRC2 ,IRS1
5	SMALL_GTPASE_MEDIATED_SIGNAL_TRANSDUCTION	06//14	89/75605	6.669760280615991E-15	CCL5 , CXCL5 ,NET1 , GDF15 , BIRC2 , IRS1
6	MAPKKK_CASCADE_GO_0000165	06//14	104/75605	1.738836352936306 6E-14	CCL5 , CXCL5 , NET1 ,GDF15 , BIRC2 ,IRS1
7	ACTIN_CYTOSKELETON_ORGANIZATION_AND_BIOGENESIS	06//14	105/75605	1.844024962911 6683E-14	CCL5 , CXCL5 , NET1 , GDF15 , BIRC2, IRS1
8	ACTIN_FILAMENT_BASED_PROCESS	06//14	115/75605	3.220623649423951E-14	CCL5 ,CXCL5 , NET1 ,GDF15 , BIRC2 ,IRS1
9	NEGATIVE_REGULATION_OF_SIGNAL_TRANSDUCTION	05//14	37/75605	4.223564796025843E-14	CCL5 , NET1 , GDF15 , BIRC2 , IRS1
10	ACTIVATION_OF_MAPK_ACTIVITY	05//14	41/75605	7.257731545328 4E-14	CCL5 , CXCL5, GDF15,BIRC2 ,IRS1
11	POSITIVE_REGULATION_OF_MAP_KINASE_ACTIVITY	05//14	47/75605	1.484520068315 7144E-13	CCL5 ,CXCL5 , GDF15 ,BIRC2 ,IRS1
12	JNK_CASCADE	05//14	47/75605	1.484520068315 7144E-13	CCL5 , NET1 , GDF15 , BIRC2 ,IRS1
13	PHOSPHOINOSITIDE_MEDIATED_SIGNALING	05//14	48/75605	1.656941292582 25E-13	CCL5 , NET1 , GDF15 , BIRC2 , IRS1
14	SECOND_MESSENGER_MEDIATED_SIGNALING	06//14	153/75605	1.839191407306 9062E-13	CCL5 ,CXCL5 , NET1 , GDF15 , BIRC2 , IRS1
15	STRESS_ACTIVATED_PROTEIN_KINASE_SIGNALING_PATHWAY	05//14	49/75605	1.845010280712024E-13	CCL5 , NET1 , GDF15, BIRC2, IRS1
16	NEGATIVE_REGULATION_OF_CELL_PROLIFERATION	06//14	156/75605	2.069791279753352 3E-13	CCL5 ,CXCL5 , NET1 , GDF15 , BIRC2 ,IRS1
17	EMBRYONIC_DEVELOPMENT	05//14	58/75605	4.428686683789125E-13	CCL5 ,CXCL5 , NET1 , GDF15 , IRS1
18	POSITIVE_REGULATION_OF_T_CELL_PROLIFERATION	04//14	13/75605	5.251298240323559E-13	CCL5 , CXCL5, GDF15 , IRS1
19	REGULATION_OF_CELL_CYCLE	06//14	182/75605	5.278479017407478E-13	CCL5 , CXCL5 , NET1 , GDF15 , BIRC2 ,IRS1
20	POSITIVE_REGULATION_OF_MULTICELLULAR_ORGANISMAL_PROCESS	05//14	66/75605	8.629451417314108E-13	CCL5 , CXCL5 , NET1 ,GDF15 ,IRS1
21	REGULATION_OF_MAP_KINASE_ACTIVITY	05//14	67/75605	9.324263859538934E-13	CCL5 , CXCL5 , GDF15 , BIRC2 , IRS1
22	CYTOSKELETON_ORGANIZATION_AND_BIOGENESIS	06//14	208/75605	1.185285710153952E-12	CCL5 , CXCL5 , NET1,GDF15 , BIRC2 , IRS1
23	REGULATION_OF_T_CELL_PROLIFERATION	04//14	16/75605	1.336163670673344 5E-12	CCL5 , CXCL5 , GDF15 , IRS1
24	NEGATIVE_REGULATION_OF_CELL_CYCLE	05//14	79/75605	2.172842259085167E-12	CCL5 ,CXCL5 , NET1 , GDF15 , IRS1
25	IMMUNE_RESPONSE	06//14	235/75605	2.478934547628 1695E-12	CCL5 ,CXCL5 , NET1 , GDF15 , BIRC2 , IRS1
26	T_CELL_PROLIFERATION	04//14	19/75605	2.844458893133801E-12	CCL5 ,CXCL5 , GDF15, IRS1
27	POSITIVE_REGULATION_OF_TRANSFERASE_ACTIVITY	05//14	86/75605	3.354804597241 54E-12	CCL5 , CXCL5 , GDF15 , BIRC2 , IRS1
28	POSITIVE_REGULATION_OF_T_CELL_ACTIVATION	04//14	21/75605	4.391017075577413E-12	CCL5 ,CXCL5 , GDF15 , IRS1
29	EPIDERMAL_GROWTH_FACTOR_RECEPTOR_SIGNALING_PATHWAY	04//14	22/75605	5.366088603586 4344E-12	CCL5 , GDF15 , BIRC2 , IRS1
30	POSITIVE_REGULATION_OF_LYMPHOCYTE_ACTIVATION	04//14	24/75605	7.792887262315483E-12	CCL5, CXCL5 , GDF15 , IRS1
31	PROTEIN_KINASE_CASCADE	06//14	293/75605	9.374640213601346E-12	CCL5 , CXCL5 ,NET1 , GDF15 , BIRC2, IRS1
32	REGULATION_OF_CELL_PROLIFERATION	06//14	308/75605	1.266068769454587E-11	CCL5 , CXCL5 , NET1 , GDF15 , BIRC2 , IRS1
33	CELL_CYCLE_GO_0007049	06//14	315/75605	1.449335461380422 5E-11	CCL5 , CXCL5 , NET1 , GDF15 , BIRC2 , IRS1
34	ACTIVATION_OF_PROTEIN_KINASE_ACTIVITY	04//14	28/75605	1.500799337733989E-11	CCL5 , GDF15 , BIRC2 , IRS1
35	REGULATION_OF_T_CELL_ACTIVATION	04//14	28/75605	1.500799337733989E-11	CCL5 , CXCL5 ,GDF15, IRS1
36	IMMUNE_SYSTEM_PROCESS	06//14	332/75605	1.980019323571278E-11	CCL5 , CXCL5 , NET1 , GDF15 , BIRC2 , IRS1
37	PATTERN_SPECIFICATION_PROCESS	04//14	31/75605	2.305441198677 21E-11	CXCL5 , GDF15 ,BIRC2 ,IRS1
38	G_PROTEIN_COUPLED_RECEPTOR_PROTEIN_SIGNALING_PATHWAY	06//14	342/75605	2.376113445003917 6E-11	CCL5 , CXCL5 ,NET1 , GDF15 ,BIRC2 , IRS1
39	CELLULAR_PROTEIN_COMPLEX_ASSEMBLY	04//14	33/75605	2.997415840978 33E-11	CCL5 , GDF15, BIRC2 , IRS1
40	RECEPTOR_MEDIATED_ENDOCYTOSIS	04//14	33/75605	2.997415840978 33E-11	CCL5 ,GDF15 ,BIRC2 , IRS1
41	MEMBRANE_ORGANIZATION_AND_BIOGENESIS	05//14	135/75605	3.320297490145723E-11	CCL5 ,GDF15 , BIRC2 ,MYO9A , IRS1
42	REGULATION_OF_LYMPHOCYTE_ACTIVATION	04//14	35/75605	3.834388092901063 4E-11	CCL5,CXCL5,GDF15, IRS1
43	SEXUAL_REPRODUCTION	05//14	139/75605	3.848766054769965E-11	CCL5 ,CXCL5,NET1 ,GDF15 , IRS1
44	NEUROLOGICAL_SYSTEM_PROCESS	06//14	379/75605	4.402668836698638E-11	CCL5 ,CXCL5,NET1 ,GDF15 ,MYO9A,IRS1
45	POSITIVE_REGULATION_OF_TRANSCRIPTION	05//14	144/75605	4.601605834862667 4E-11	CCL5 , GDF15 ,BIRC2 ,EGR1 ,IRS1
46	POSITIVE_REGULATION_OF_CELL_PROLIFERATION	05//14	149/75605	5.467673365969297E-11	CCL5 , CXCL5 , GDF15, BIRC2 , IRS1
47	REGULATION_OF_MULTICELLULAR_ORGANISMAL_PROCESS	05//14	151/75605	5.848524358151822E-11	CCL5 , CXCL5 , NET1 ,GDF15 , IRS1
48	POSITIVE_REGULATION_OF_NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_A	05//14	154/75605	6.459238302750 46E-11	CCL5 , GDF15 , BIRC2 , EGR1, IRS1
49	REGULATION_OF_PROTEIN_KINASE_ACTIVITY	05//14	155/75605	6.673750087014214E-11	CCL5 ,CXCL5 , GDF15, BIRC2, IRS1
50	PROTEIN_UBIQUITINATION	04//14	40/75605	6.688176635772876E-11	CCL5 , GDF15 , BIRC2 , IRS1

MF up core

Rank	Name	List ratio	BG ratio	P-value	Genes
1	ENZYME_BINDING	13//35	178/23685	1.9775859654661493E-19	FOXA3, LOX , ARHGEF3, VEGFA , RYBP , KLF10 ,NT5E , ID1 , MAP3K13 , NOX4 , BCAR1 ,BNIP3 , RASA2
2	ENZYME_ACTIVATOR_ACTIVITY	10//35	125/23685	1.8865005888381997E-15	LOX,CXCL12 ,ARHGEF3 , VEGFA, MAP3K13 , NOX4 , BCAR1 , CCL3 , BNIP3 , RASA2
3	PROTEIN_C_TERMINUS_BINDING	09//35	73/23685	1.1972673859365575E-13	FOXA3, KLF10 , NT5E , MAP3K13 , NOX4 ,BCAR1 , FOSB , BNIP3
4	RECEPTOR_BINDING	12//35	377/23685	1.29696546260647E-13	CXCL12 , ARHGEF3 , VEGFA , RYBP , KRT19 , MAP3K13 , NOX4 , THBS1 , BCAR1 ,CCL3 , BNIP3 , RASA2
5	ENZYME_REGULATOR_ACTIVITY	11//35	323/23685	7.7676220195230 35E-13	LOX , CXCL12 , ARHGEF3 ,VEGFA , MAP3K13 , NOX4 , THBS1 , BCAR1 , CCL3 , BNIP3 , RASA2
6	CYTOSKELETAL_PROTEIN_BINDING	09//35	159/23685	1.3179194790624777E-12	LOX , ARHGEF3 , VEGFA ,NT5E , KRT19 , MAP3K13 , NOX4 , BCAR1 , BNIP3
7	SEQUENCE_SPECIFIC_DNA_BINDING	07//35	58/23685	2.2962757879386 69E-12	GLIS3 , RYBP , ID1 , MAP3K13 , NOX4 , BCAR1 , BNIP3
8	KINASE_ACTIVATOR_ACTIVITY	05//35	12/23685	4.1044958842551 84E-12	CXCL12 , ARHGEF3 , MAP3K13 , BCAR1 , CCL3
9	CHROMATIN_BINDING	06//35	32/23685	5.814387 13520864E-12	ARHGEF3 ,VEGFA , NT5E , ID1 , MAP3K13 , BCAR1
10	PHOSPHATASE_BINDING	05//35	13/23685	6.6613533904659135E-12	LOX , NT5E , MAP3K13 ,NOX4 ,BCAR1
11	TRANSCRIPTION_FACTOR_BINDING	10//35	307/23685	1.5482371720682472E-11	GLIS3 , FOXA3 , RYBP , KLF10 , ID1 , MAP3K13 , NOX4 , BCAR1 ,FOSB , BNIP3
12	TUBULIN_BINDING	06//35	47/23685	6.7638740996047 78E-11	LOX ,ARHGEF3, MAP3K13 , NOX4 ,BCAR1 , BNIP3
13	IDENTICAL_PROTEIN_BINDING	09//35	304/23685	4.2809980855920 63E-10	LOX , ARHGEF3 , VEGFA, NT5E, MAP3K13 ,NOX4 ,THBS1 ,BCAR1 , BNIP3
14	PROTEIN_HOMODIMERIZATION_ACTIVITY	07//35	121/23685	4.4745391364055173E-10	LOX ,ARHGEF3 , VEGFA ,MAP3K13 , NOX4 ,BCAR1 , BNIP3
15	TRANSCRIPTION_COFACTOR_ACTIVITY	08//35	228/23685	1.1925634 19240081E-9	GLIS3 ,FOXA3 , RYBP , KLF10 , MAP3K13 , BCAR1 , FOSB , BNIP3
16	MICROTUBULE_BINDING	05//35	33/23685	1.1976456374687557E-9	LOX , ARHGEF3 ,MAP3K13 , BCAR1 , BNIP3
17	LOW_DENSITY_LIPOPROTEIN_BINDING	04//35	12/23685	1.9565039555064636E-9	LOX, CXCL12 ,VEGFA, CCL3
18	RECEPTOR_SIGNALING_PROTEIN_ACTIVITY	06//35	82/23685	2.112929277 17694E-9	CXCL12 , RYBP , MAP3K13 , NOX4 ,BCAR1 , CCL3
19	PROTEIN_KINASE_REGULATOR_ACTIVITY	05//35	39/23685	2.8833433685708 82E-9	CXCL12 , ARHGEF3 , MAP3K13 , BCAR1 ,CCL3
20	KINASE_REGULATOR_ACTIVITY	05//35	46/23685	6.8038816951161 11E-9	CXCL12 , ARHGEF3 , MAP3K13 , BCAR1 , CCL3
21	PROTEIN_DIMERIZATION_ACTIVITY	07//35	182/23685	7.6958110628338 23E-9	LOX ,ARHGEF3 , VEGFA , MAP3K13, NOX4 , BCAR1 , BNIP3
22	LIPOPROTEIN_BINDING	04//35	18/23685	1.2000023215928735E-8	LOX , CXCL12 , VEGFA , CCL3
23	TRANSITION_METAL_ION_BINDING	06//35	111/23685	1.3183968537362485E-8	LOX ,VEGFA , NT5E , MAP3K13 , NOX4 , BCAR1
24	CYTOKINE_ACTIVITY	06//35	113/23685	1.4675191920895845E-8	CXCL12 , VEGFA , MAP3K13 , BCAR1 , CCL3 , BNIP3
25	TRANSCRIPTION_FACTOR_ACTIVITY	08//35	354/23685	3.6405787093921386E-8	GLIS3 , FOXA3 , RYBP , KLF10 , ID1 , MAP3K13 , BCAR1 , BNIP3
26	TRANSCRIPTION_REPRESSOR_ACTIVITY	06//35	152/23685	8.5808660303711 1E-8	GLIS3 , RYBP , ID1 , MAP3K13 , BCAR1 , BNIP3
27	PROTEIN_HETERODIMERIZATION_ACTIVITY	05//35	77/23685	9.428194184346 72E-8	LOX , MAP3K13 , NOX4 , BCAR1 , BNIP3
28	OXIDOREDUCTASE_ACTIVITY	07//35	289/23685	1.8007010886633444E-7	LOX , NT5E ,MAP3K13 ,NOX4 , BCAR1, BNIP3 , RASA2
29	MAP_KINASE_KINASE_KINASE_ACTIVITY	03//35	10/23685	3.5137094588948713E-7	MAP3K13 , NOX4 ,BCAR1
30	PROTEIN_PHOSPHATASE_BINDING	03//35	10/23685	3.5137094588948713E-7	LOX , MAP3K13 , BCAR1
31	PHOSPHOTRANSFERASE_ACTIVITY_ALCOHOL_GROUP_AS_ACCE	07//35	334/23685	4.745358742107 03E-7	CXCL12 , VEGFA , NT5E , MAP3K13 , NOX4 , BCAR1, CCL3
32	NF_KAPPAB_BINDING	03//35	11/23685	4.8248202749639 84E-7	MAP3K13 , BCAR1 , BNIP3
33	CATION_BINDING	06//35	213/23685	6.2025832977832 98E-7	LOX ,VEGFA , NT5E , MAP3K13 , NOX4 , BCAR1
34	PHOSPHOLIPID_BINDING	04//35	47/23685	6.733686049867 98E-7	LOX , VEGFA , NOX4 , RASA2
35	ENZYME_INHIBITOR_ACTIVITY	05//35	119/23685	8.2616168510856 82E-7	VEGFA , MAP3K13 , THBS1 , BCAR1 , BNIP3
36	KINASE_ACTIVITY	07//35	369/23685	9.1960218696183 24E-7	CXCL12 , VEGFA , NT5E , MAP3K13 , NOX4 , BCAR1 , CCL3
37	NUCLEASE_ACTIVITY	04//35	55/23685	1.2741118202272643E-6	VEGFA , NT5E , NOX4 , BNIP3
38	PROTEIN_KINASE_BINDING	04//35	62/23685	2.0649298965934 94E-6	NT5E , MAP3K13 , NOX4 , BCAR1
39	ION_BINDING	06//35	273/23685	2.5932377310941 847E-6	LOX , VEGFA , NT5E , MAP3K13 , NOX4 , BCAR1
40	KINASE_BINDING	04//35	70/23685	3.3585233491262257E-6	NT5E,MAP3K13 , NOX4 , BCAR1
41	COFACTOR_BINDING	03//35	22/23685	4.4366464110986 28E-6	LOX , NOX4 , BNIP3
42	ACTIN_BINDING	04//35	76/23685	4.6625520841017 37E-6	VEGFA , MAP3K13 , NOX4 , BCAR1
43	SERINE_TYPE_ENDOPEPTIDASE_INHIBITOR_ACTIVITY	03//35	25/23685	6.5993133174082 97E-6	VEGFA ,THBS1 , BNIP3
44	CALMODULIN_BINDING	03//35	25/23685	6.5993133174082 97E-6	KRT19 , NOX4 , BNIP3
45	OXIDOREDUCTASE_ACTIVITY_ACTING_ON_NADH_OR_NADPH	03//35	25/23685	6.5993133174082 97E-6	MAP3K13 , NOX4 , BCAR1
46	LIPID_BINDING	04//35	87/23685	7.9731866111294447E-6	LOX , VEGFA, NOX4 , RASA2
47	TRANSCRIPTION_COREPRESSOR_ACTIVITY	04//35	94/23685	1.0823289165556684E-5	GLIS3 , RYBP , MAP3K13, BCAR1
48	GROWTH_FACTOR_BINDING	03//35	32/23685	1.4097354456996228E-5	NOX4 , CTGF , BNIP3
49	RECEPTOR_SIGNALING_PROTEIN_SERINE_THREONINE_KINASE	03//35	34/23685	1.6961786746298 31E-5	MAP3K13 , NOX4 , BCAR1
50	PROTEIN_N_TERMINUS_BINDING	03//35	38/23685	2.3782879086624323E-5	NT5E, MAP3K13 , BCAR1

mf down core

Rank	Name	List ratio	BG ratio	P-value	Genes
1	KINASE_ACTIVATOR_ACTIVITY	04//14	12/23685	3.7670319955620004E-11	CCL5 , CXCL5, IRS1 , CXCL3
2	LIPOPROTEIN_BINDING	04//14	18/23685	2.3228141014688057E-10	CCL5 , CXCL5 , IRS1 , CXCL3
3	RECEPTOR_SIGNALING_PROTEIN_ACTIVITY	05//14	82/23685	8.5439456080845 92E-10	CCL5 ,CXCL5 , GDF15 ,IRS1 , CXCL3
4	PROTEIN_KINASE_REGULATOR_ACTIVITY	04//14	39/23685	6.1883978321173 07E-9	CCL5 , CXCL5 ,IRS1 ,CXCL3
5	ENZYME_ACTIVATOR_ACTIVITY	05//14	125/23685	7.2243940513133 7E-9	CCL5 ,CXCL5 , NET1 ,IRS1 , CXCL3
6	HORMONE_ACTIVITY	04//14	44/23685	1.0192048429053531E-8	CCL5 , CXCL5 , GDF15 , CXCL3
7	KINASE_REGULATOR_ACTIVITY	04//14	46/23685	1.2241402660559133E-8	CCL5 , CXCL5, IRS1 , CXCL3
8	ENZYME_REGULATOR_ACTIVITY	06//14	323/23685	1.6561039677096937E-8	CCL5 ,CXCL5 , NET1 ,IRS1 ,CXCL3 , SERPINA7
9	LOW_DENSITY_LIPOPROTEIN_BINDING	03//14	12/23685	3.6015869943943626E-8	CCL5 , CXCL5 ,CXCL3
10	NEUROPEPTIDE_HORMONE_ACTIVITY	03//14	12/23685	3.6015869943943626E-8	CCL5 ,CXCL5 , CXCL3
11	RECEPTOR_BINDING	06//14	377/23685	4.1380805388678 31E-8	CCL5 , CXCL5 , GDF15, IRS1, CXCL3 , SERPINA7
12	PROTEIN_TYROSINE_KINASE_ACTIVITY	04//14	63/23685	4.4363732759443765E-8	CCL5 , CXCL5 , IRS1 , CXCL3
13	CYTOKINE_ACTIVITY	04//14	113/23685	4.6948616990346565E-7	CCL5 , CXCL5 , GDF15 ,CXCL3
14	CHEMOKINE_ACTIVITY	03//14	42/23685	1.8533351446612269E-6	CCL5 ,CXCL5 , CXCL3
15	CHEMOKINE_RECEPTOR_BINDING	03//14	43/23685	1.9914083385040253E-6	CCL5 , CXCL5 , CXCL3
16	G_PROTEIN_COUPLED_RECEPTOR_BINDING	03//14	54/23685	3.9820616577550 71E-6	CCL5 ,CXCL5 , CXCL3
17	PROTEIN_HETERODIMERIZATION_ACTIVITY	03//14	77/23685	1.1618435795905316E-5	NET1 , GDF15 , IRS1
18	PROTEIN_KINASE_ACTIVITY	04//14	285/23685	1.8238855023254052E-5	CCL5 , CXCL5 , IRS1 , CXCL3
19	PHOSPHOTRANSFERASE_ACTIVITY_ALCOHOL_GROUP_AS_ACCEPTOR	04//14	334/23685	3.3794818348217 59E-5	CCL5 , CXCL5 , IRS1 ,CXCL3
20	KINASE_ACTIVITY	04//14	369/23685	4.9681997026179195E-5	CCL5 ,CXCL5, IRS1, CXCL3
21	ENZYME_BINDING	03//14	178/23685	1.4002377716995062E-4	NET1 , EGR1 ,IRS1
22	PROTEIN_DIMERIZATION_ACTIVITY	03//14	182/23685	1.4945318439874 37E-4	NET1 ,GDF15 , IRS1
23	SMALL_GTPASE_BINDING	02//14	33/23685	1.6863555504640558E-4	NET1, IRS1
24	GTPASE_BINDING	02//14	34/23685	1.7908437145411 02E-4	NET1, IRS1
25	SH3_SH2_ADAPTOR_ACTIVITY	02//14	43/23685	2.8694494175045103E-4	NET1 ,IRS1
26	TUBULIN_BINDING	02//14	47/23685	3.4281080003529 83E-4	NET1 ,IRS1
27	MOLECULAR_ADAPTOR_ACTIVITY	02//14	49/23685	3.7255899786745896E-4	NET1 , IRS1
28	PROTEIN_BINDING_BRIDGING	02//14	59/23685	5.3930177651910 48E-4	NET1 ,IRS1
29	GTPASE_ACTIVATOR_ACTIVITY	02//14	60/23685	5.5761502452889 02E-4	NET1, IRS1
30	IDENTICAL_PROTEIN_BINDING	03//14	304/23685	6.6212220092166 5E-4	NET1 , IRS1,SERPINA7
31	TRANSCRIPTION_FACTOR_BINDING	03//14	307/23685	6.8102256414775 92E-4	GDF15 , EGR1
32	SMALL_GTPASE_REGULATOR_ACTIVITY	02//14	67/23685	6.9407307385762 15E-4	NET1 , IRS1
33	PROTEIN_C_TERMINUS_BINDING	02//14	73/23685	8.2246491687425 7E-4	EGR1 ,IRS1
34	ACTIN_BINDING	02//14	76/23685	8.9058328149128 61E-4	NET1 , IRS1
35	ENZYME_INHIBITOR_ACTIVITY	02//14	119/23685	0.0021464748739 29441	NET1 ,SERPINA7
36	GTPASE_REGULATOR_ACTIVITY	02//14	125/23685	0.0023621157857 99148	NET1
37	CYTOSKELETAL_PROTEIN_BINDING	02//14	159/23685	0.0037626655320047338	NET1
38	BETA_TUBULIN_BINDING	01//14	10/23685	0.0058963337044055	NET1
39	STEROID_HORMONE_RECEPTOR_BINDING	01//14	10/23685	0.0058963337044055	IRS1
40	INSULIN_LIKE_GROWTH_FACTOR_RECEPTOR_BINDING	01//14	10/23685	0.0058963337044055	IRS1
41	ENDODEOXYRIBONUCLEASE_ACTIVITY	01//14	11/23685	0.0064841880371674 14	IRS1
42	UBIQUITIN_BINDING	01//14	11/23685	0.0064841880371674 14	IRS1
43	INOSITOL_OR_PHOSPHATIDYLINOSITOL_PHOSPHATASE_ACTIVITY	01//14	12/23685	0.0070717195640525 91	IRS1
44	SMALL_CONJUGATING_PROTEIN_BINDING	01//14	12/23685	0.0070717195640525 91	IRS1
45	SMAD_BINDING	01//14	12/23685	0.0070717195640525 91	IRS1
46	TRANSCRIPTION_COFACTOR_ACTIVITY	01//14	228/23685	0.0074832632766658 14	EGR1
47	RNA_POLYMERASE_II_TRANSCRIPTION_MEDIATOR_ACTIVITY	01//14	13/23685	0.0076589284486934 58	IRS1
48	HORMONE_BINDING	01//14	13/23685	0.0076589284486934 58	IRS1
49	PDZ_DOMAIN_BINDING	01//14	14/23685	0.0082458148546464 12	IRS1
50	COLLAGEN_BINDING	01//14	14/23685	0.0082458148546464 12	IRS1

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Rank	Name	List ratio	BG ratio	P-value	Genes
1	CELL_PROJECTION	15/35	108/35110	2.32938614065639O6E-29	EMP1 , FOXA3 ,FHL2 , IL15 , KRT19 , LGALS1 , NOX4 , GLIS3 , CD63 , VEGFA , KLF10 , MAP3K13 , CLIC4 ,BCAR1 , BNIP3
2	LAMELLIPODIUM	11/35	25/35110	7.3686884557755O4E-28	GLIS3, FOXA3 ,FHL2, CD63 , IL15 , KLF10, KRT19, MAP3K13 , LGALS1 , CLIC4 , BCAR1
3	SYNAPSE	11/35	27/35110	2.1523936046337446E-27	GLIS3 , FOXA3 ,FHL2 , CD63 , IL15 , VEGFA, KLF10 , KRT19 ,MAP3K13 , CTGF,BNIP3
4	LEADING_EDGE	12/35	47/35110	5.827921650093961E-27	GLIS3 , FOXA3 , FHL2 ,CD63 , IL15 , KLF10 , KRT19 , MAP3K13 , LGALS1,CLIC4 , BCAR1, BNIP3
5	SOLUBLE_FRACTION	15/35	161/35110	1.2748836632725625E-26	FOXA3 , FHL2, IL15 , NT5E , KRT19 , LGALS1 , CTGF, GLIS3 , CD63 , VEGFA , KLF10 , MAP3K13 , CLIC4, BNIP3 , CCL3
6	SITE_OF_POLARIZED_GROWTH	8//35	11/35110	6.770659328611517E-23	GLIS3 ,FOXA3 , FHL2 , CD63 ,IL15 ,KLF10 , KRT19 ,MAP3K13
7	PERINUCLEAR_REGION_OF_CYTOPLASM	10//35	54/35110	5.43459804630688SE-21	EMP1,CD63 ,IL15 , VEGFA,KRT19 ,MAP3K13 ,NOX4 ,CTGF , BCAR1 , BNIP3
8	GROWTH_CONE	7//35	10/35110	6.172730972337454E-20	GLIS3 , FOXA3 ,FHL2 , CD63,IL15 , KLF10 , KRT19
9	MEMBRANE_FRACTION	14//35	339/35110	8.919431882973318E-20	EMP1, IL15 , NT5E ,KRT19, LGALS1 , NOX4, CTGF ,CD63 , VEGFA, MAP3K13 , CLIC4 , BCAR1,BNIP3 , CCL3
10	ER_GOLGI_INTERMEDIATE_COMPARTMENT	8//35	24/35110	2.98790708553797SE-19	EMP1 , CD63, IL15 , VEGFA , KRT19 , MAP3K13 , NOX4, BNIP3
11	RUFFLE	8//35	31/35110	3.1876292945071816E-18	CD63 , IL15 , KRT19 , MAP3K13 , LGALS1 , CLIC4 , BCAR1 , BNIP3
12	BASOLATERAL_PLASMA_MEMBRANE	8//35	35/35110	9.480967628599661E-18	EMP1 ,CD63 ,IL15 , KRT19 , NOX4 , CTGF , BCAR1 ,BNIP3
13	APICAL_PART_OF_CELL	7//35	17/35110	9.94820563387466SE-18	CD63 , IL15 ,KRT19 , MAP3K13 , LGALS1 , CLIC4 , BCAR1
14	MICROVILLUS	6//35	11/35110	2.871717047950448E-16	CD63 ,KRT19, MAP3K13 , LGALS1, CLIC4,BNIP3
15	IMMUNOLOGICAL_SYNAPSE	6//35	11/35110	2.871717047950448E-16	EMP1 ,CD63 ,IL15 , KRT19 , NOX4 , BNIP3
16	LIPID_RAFT	7//35	29/35110	7.907715006831736E-16	EMP1 , CD63 , IL15 , KRT19 , MAP3K13 , NOX4 , BNIP3
17	CYTOSKELETON	12//35	367/35110	9.387102038600843E-16	EMP1 ,CD63 , IL15 , VEGFA , KRT19 ,MAP3K13 , NOX4 , LGALS1, CTGF , CLIC4 , BCAR1 , BNIP3
18	LYSOSOME	8//35	61/35110	1.16274558615032395-15	EMP1 , CD63 , IL15 , VEGFA , KRT19 , MAP3K13 , NOX4 , BNIP3
19	LYTIC_VACUOLE	8//35	61/35110	1.16274558615032395-15	EMP1 , CD63 , IL15 , VEGFA , KRT19 , MAP3K13 , NOX4 , BNIP3
20	INSOLUBLE_FRACTION	6//35	15/35110	3.100757350326135E-15	CD63 , IL15 , KRT19, LGALS1 , CLIC4 , BNIP3
21	VACUOLE	8//35	69/35110	3.281170656207309SE-15	EMP1 , CD63 , IL15 , VEGFA , KRT19 , MAP3K13 , NOX4 , BNIP3
22	DENDRITE	6//35	16/35110	4.957112169759517E-15	CD63 , IL15 , KRT19 , MAP3K13, CTGF , BNIP3
23	ACTIN_CYTOSKELETON	9//35	129/35110	5.930504334208202E-15	CD63 , IL15 , VEGFA , KRT19 , MAP3K13 , LGALS1, CLIC4 , BCAR1 , BNIP3
24	CONTRACTILE_FIBER_PART	6//35	23/35110	6.2127615311093 14E-14	CD63 , KRT19 , MAP3K13 ,CTGF , BCAR1 , BNIP3
25	CONTRACTILE_FIBER	6//35	25/35110	1.088157144425934 6E-13	CD63 , KRT19 ,MAP3K13 , CTGF ,BCAR1 , BNIP3
26	ENDOSOME	7//35	67/35110	4.274335812577565E-13	EMP1 ,CD63 , IL15 , KRT19 , MAP3K13 , NOX4 , BNIP3
27	SYNAPSE_PART	5//35	13/35110	9.335766823831846E-13	CD63 , IL15 , KRT19 , CTGF, BNIP3
28	PORE_COMPLEX	6//35	36/35110	1.185945533925678 3E-12	EMP1 ,CD63 , VEGFA,KRT19 , NOX4 , BNIP3
29	APICAL_PLASMA_MEMBRANE	5//35	14/35110	1.450989066191691 2E-12	CD63,IL15 , KRT19, MAP3K13, BCAR1
30	CELL_MATRIX_JUNCTION	5//35	18/35110	6.188621037286018E-12	CD63 , IL15 , KRT19 , CTGF , BCAR1
31	PEROXISOME	6//35	47/35110	6.478525394002783E-12	CD63 , IL15 , VEGFA , KRT19 , MAP3K13, BNIP3
32	MICROBODY	6//35	47/35110	6.478525394002783E-12	CD63 , IL15 ,VEGFA ,KRT19 ,MAP3K13 , BNIP3
33	MYOFIBRIL	5//35	19/35110	8.39166270184427E-12	CD63 ,KRT19 , MAP3K13 ,CTGF , BCAR1
34	CORTICAL_CYTOSKELETON	5//35	20/35110	1.117931800099747 1E-11	CD63 , IL15 , KRT19 , MAP3K13 , BNIP3 A
35	RECEPTOR_COMPLEX	6//35	56/35110	1.944450632784827 3E-11	EMP1 , CD63 , IL15 , KRT19 , NOX4 , BNIP3
36	CYTOSOL	8//35	205/35110	2.380051616827993 6E-11	EMP1 ,CD63 ,IL15 , VEGFA , KRT19 , MAP3K13 , NOX4 , BNIP3
37	CELL_CORTEX_PART	5//35	24/35110	3.054326525494061E-11	CD63 , IL15 ,KRT19 ,MAP3K13 , BNIP3
38	UBIQUITIN_LIGASE_COMPLEX	5//35	26/35110	4.718853796226579 4E-11	CD63 , KRT19 ,MAP3K13 , LGALS1 , CLIC4
39	INTERCELLULAR_JUNCTION	6//35	65/35110	4.909919775041671E-11	CD63 , IL15 , KRT19 ,CTGF , BCAR1, BNIP3
40	GOLGI_APPARATUS	8//35	225/35110	4.996792859633175E-11	EMP1 CD63 ,IL15 , VEGFA, KRT19 , MAP3K13 ,NOX4 , BNIP3
41	INTERCALATED_DISC	4//35	9/35110	1.037561559396540 9E-10	CD63 , IL15 ,KRT19 ,BCAR1
42	TRANSPORT_VESICLE	5//35	33/35110	1.692409321726682E-10	CD63 , IL15 , KRT19 , CTGF , BNIP3
43	CELL_SOMA	4//35	10/35110	1.727742034359172E-10	CD63, KRT19 ,MAP3K13 , BNIP3
44	CELL_JUNCTION	6//35	82/35110	2.052369727329431 2E-10	CD63 , IL15 , KRT19 , CTGF , BCAR1 , BNIP3
45	LATE_ENDOSOME	4//35	12/35110	4.065344218130752 5E-10	CD63 , IL15 ,KRT19,BNIP3
46	CELL_CORTEX	5//35	39/35110	4.084616551624752 6E-10	CD63 , IL15 , KRT19 , MAP3K13 , BNIP3
47	ENDOPLASMIC_RETICULUM	8//35	294/35110	4.146901436340496 3E-10	EMP1 , CD63 , IL15 ,VEGFA, KRT19 , MAP3K13 , NOX4 ,BNIP3
48	FOCAL_ADHESION	4//35	13/35110	5.866977333600762E-10	CD63 , IL15 , KRT19 , BCAR1
49	ENDOCYTIC_VESICLE	4//35	14/35110	8.206513321740025E-10	CD63 , IL15 , KRT19 , BNIP3
50	VOLTAGE_GATED_CALCIUM_CHANNEL_COMPLEX	4//35	15/35110	1.118081533417063 8E-9	EMP1 , CD63, KRT19 , NOX4

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Rank	Name	List ratio	BG ratio	P-value	Genes
1	SYNAPSE	04/14	27/35110	2.756936266585131E-10	GDF15,EGR1 , CXCL3 , SERPINA7
2	SOLUBLE_FRACTION	05//14	161/35110	3.663729048245001 3E-9	GDF15 ,EGR1 , IRS1 , CXCL3 , SERPINA7
3	NEURON_PROJECTION	03//14	21/35110	6.674189617504483E-8	GDF15 , CXCL3 , SERPINA7
4	CELL_PROJECTION	04//14	108/35110	8.226235574040378E-8	GDF15 , EGR1 , CXCL3 , SERPINA7
5	ER_GOLGI_INTERMEDIATE_COMPARTMENT	03//14	24/35110	1.014726245782539E-7	GDF15 , CXCL3 , SERPINA7
6	PORE_COMPLEX	03//14	36/35110	3.566171203293274E-7	GDF15 A, CXCL3,SERPINA7
7	MICROBODY	03//14	47/35110	8.070904917855583E-7	GDF15 , CXCL3 , SERPINA7
8	PEROXISOME	03//14	47/35110	8.070904917855583E-7	GDF15 ,CXCL3 , SERPINA7
9	CYTOSOL	04//14	205/35110	1.066783150158533 8E-6	GDF15 , IRS1 , CXCL3 SERPINA7
10	PERINUCLEAR_REGION_OF_CYTOPLASM	03//14	54/35110	1.231893144148259E-6	GDF15 , CXCL3, SERPINA7
11	GOLGI_APPARATUS	04//14	225/35110	1.543276041733514 1E-6	GDF15 , IRS1 , CXCL3 ,SERPINA7
12	LYTIC_VACUOLE	03//14	61/35110	1.783524211380715 3E-6	GDF15 ,CXCL3 , SERPINA7
13	LYSOSOME	03//14	61/35110	1.783524211380715 3E-6	GDF15 , CXCL3, SERPINA7
14	VACUOLE	03//14	69/35110	2.589929989358981E-6	GDF15 , CXCL3 , SERPINA7
15	ENDOPLASMIC_RETICULUM	04//14	294/35110	4.438603840329756E-6	GDF15, IRS1 , CXCL3 , SERPINA7
16	MEMBRANE_FRACTION	04//14	339/35110	7.766467275360192E-6	GDF15 , IRS1 , CXCL3 , SERPINA7
17	EXTRACELLULAR_MATRIX	03//14	100/35110	7.915670285821521E-6	GDF15 , CXCL3, SERPINA7
18	ACTIN_CYTOSKELETON	03//14	129/35110	1.695393320210862E-5	GDF15 ,CXCL3 , SERPINA7
19	EXTRACELLULAR_SPACE	03//14	245/35110	1.132293250571902 1E-4	GDF15 , CXCL3 , SERPINA7
20	EXTRACELLULAR_REGION_PART	03//14	338/35110	2.896815232999686 5E-4	GDF15 , CXCL3 , SERPINA7
21	CYTOSKELETON	03//14	367/35110	3.676956202313449 3E-4	GDF15 , CXCL3 , SERPINA7
22	GROWTH_CONE	01//14	10/35110	0.003980829934104174	EGR1
23	SITE_OF_POLARIZED_GROWTH	01//14	11/35110	0.00437810253754926	EGR1
24	LAMELLIPODIUM	01//14	25/35110	0.009924491924058506	EGR1
25	BASOLATERAL_PLASMA_MEMBRANE	01//14	35/35110	0.013868617871151253	IRS1
26	MICROSOME	01//14	42/35110	0.016620819677483415	IRS1
27	VESICULAR_FRACTION	01//14	44/35110	0.017405851993196525	IRS1
28	LEADING_EDGE	01//14	47/35110	0.018582309413699278	EGR1

network

node1	node2	homology	coexpression	experimental	knowledge	textmining	combined_score
Cxcl12	Cxcl5	0.000	0.000	0.000	0.900	0.568	**0.953**
Cxcl12	Ccl5	0.828	0.000	0.000	0.900	0.771	**0.912**
Vegfa	Egr1	0.000	0.000	0.000	0.000	0.890	**0.890**
Cxcl3	Cxcl5	0.905	0.275	0.000	0.900	0.704	**0.927**
Birc2	Vegfa	0.000	0.000	0.000	0.000	0.441	**0.441**
Cxcl12	Cxcl3	0.000	0.000	0.000	0.900	0.369	**0.932**
Vegfa	Ccl5	0.000	0.000	0.000	0.000	0.400	**0.400**
Lgals1	Fosb	0.000	0.000	0.000	0.000	0.806	**0.806**
Akt3	Irs1	0.000	0.000	0.000	0.000	0.487	**0.486**
Ccl5	Cxcl5	0.000	0.000	0.000	0.900	0.641	**0.961**
Vegfa	Nox4	0.000	0.000	0.000	0.000	0.866	**0.866**
Ctgf	Col1a1	0.000	0.112	0.000	0.000	0.495	**0.521**
Lgals1	Col1a1	0.000	0.399	0.000	0.000	0.081	**0.410**
Myo9a	Net1	0.000	0.000	0.000	0.900	0.000	**0.899**
Lox	Ctgf	0.000	0.135	0.000	0.000	0.471	**0.511**
Vegfa	Thbs1	0.000	0.000	0.096	0.000	0.952	**0.954**
Ccl5	Ccl3	0.930	0.352	0.000	0.900	0.456	**0.932**
Cxcl12	Egr1	0.000	0.000	0.000	0.000	0.830	**0.830**
Vegfa	Ctgf	0.000	0.000	0.072	0.000	0.929	**0.930**
Ctgf	Tgfb1	0.000	0.000	0.000	0.000	0.943	**0.943**
Vegfa	Tgfb1	0.000	0.000	0.000	0.900	0.943	**0.993**
Tgfb1	Col1a1	0.000	0.000	0.000	0.000	0.455	**0.455**
Cxcl12	Tgfb1	0.000	0.000	0.000	0.000	0.440	**0.440**
Bcar1	Irs1	0.000	0.000	0.000	0.293	0.244	**0.430**
Lgals1	Cd63	0.000	0.461	0.000	0.000	0.199	**0.539**
Ccl5	Cxcl3	0.000	0.000	0.000	0.900	0.475	**0.943**
Egr1	Fosb	0.000	0.397	0.000	0.000	0.425	**0.630**
Vegfa	Irs1	0.000	0.000	0.000	0.000	0.880	**0.880**
Irs1	Foxa2	0.000	0.000	0.000	0.000	0.845	**0.845**
Myo9a	Arhgef3	0.000	0.000	0.000	0.900	0.000	**0.899**
Egr1	Tgfb1	0.000	0.000	0.000	0.000	0.415	**0.415**
Vegfa	Lox	0.000	0.000	0.000	0.000	0.489	**0.489**
Lox	Tgfb1	0.000	0.000	0.000	0.000	0.400	**0.400**
Cxcl12	Vegfa	0.000	0.000	0.000	0.000	0.815	**0.815**
Lox	Col1a1	0.000	0.684	0.000	0.000	0.399	**0.797**
Bnip3	Vegfa	0.000	0.108	0.000	0.000	0.434	**0.461**
Thbs1	Tgfb1	0.000	0.000	0.000	0.000	0.619	**0.619**
Vegfa	Cxcl5	0.000	0.000	0.000	0.000	0.430	**0.430**

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Article III

« L'expression de la gamma SMA est associée à la TEM et à des marqueurs progéniteurs dans le carcinome hépatocellulaire. »

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manuscript soumis

Il est maintenant admis que la TEM est associée à la progression cancéreuse et la formation de métastases (Kalluri & Weinberg, 2009). Différents travaux ont montré que l'expression de plusieurs facteurs de transcription tels que Snail, Twist, Zeb1 et Zeb2 étaient nécessaires pour réprimer l'expression de la E-Cadhérine qui représente la première étape moléculaire de la TEM

Bien que de nombreuses études aient démontré *in vitro* le processus de la TEM dans des lignées de cellules cancéreuses, son existence *in vivo* est difficile car ce processus est transitoire et ne concerne que peu de cellules dans la masse tumorale. De plus, l'identification de la TEM dans les tissus est rendue difficile par le fait que les marqueurs sont probablement différents d'un type tumoral à l'autre.

Il a été montré que l'expression de Snail et de Twist était augmentée dans les carcinomes hépatocellulaires (CHC) suggérant que la TEM se produit dans ce type de tumeurs.

L'un des marqueurs classiquement exprimé au cours d'une TEM est l'alpha-actine musculaire lisse (αSMA). Deux SMA sont connues chez les mammifères : l'αSMA normalement exprimée dans les cellules musculaires lisses des parois vasculaires et des viscères et dans les cellules myoépithéliales et la γSMA exprimée dans les tractus gastro-intestinal et uro-génital.

Dans cette étude, nous avons caractérisé l'isoforme de SMA impliquée dans la TEM de cellules d'hépatome induite par le TGF-β et recherché l'expression de ce marqueur dans les cellules tumorales de patients adultes et pédiatriques atteint de CHC.

Notre analyse met en évidence que :

- La γSMA est l'isoforme polymérisée au cours de la TEM induite *in vitro* dans des lignées d'hépatome ou des hépatocytes tumoraux en culture primaire. Des expériences de transfection de vecteur codant pour la protéine γSMA exogène a permis de confirmer la polymérisation γSMA lors d'une TEM induite par le TGF-β ou par des vecteurs codant pour les facteurs de transcriptions Snail et Twist.
- Dans le foie normal, le pattern d'expression de l'αSMA et de la γSMA est différent : bien que l'expression des deux formes de SMA soit observée dans les artères, l'αSMA est localisée dans le compartiment non parenchymateux alors que l'expression de γSMA est située au niveau des canalicules biliaires et dans les canaux de Hering.
- Sur les coupes de CHC, l'αSMA n'est jamais exprimée alors que la γSMA est exprimée dans environ 50% des hépatocytes tumoraux.

- L'expression de γSMA est significativement corrélée avec une moins bonne différenciation des tumeurs et avec l'expression de marqueurs progéniteurs (EpCAM et Ck19).
- Cette association avec les marqueurs progéniteurs est retrouvée sur des coupes de CHC pédiatriques qui expriment tous le marqueur EpCAM. Dans ces tumeurs, l'expression de γSMA est significativement corrélée avec la perte de E-Cadhérine.
-

L'ensemble de ces résultats *in vitro* et *in vivo* suggère que l'expression de la γSMA peut représenter un marqueur fiable de la TEM dans le cancer du foie, montre une corrélation entre l'expression de cette protéine et l'agressivité de la tumeur et associe l'expression de la γSMA à un caractère souche des cellules tumorales. La γSMA pourrait constituer un marqueur potentiellement important dans le pronostic du CHC

Gamma-Smooth Muscle Actin Expression is associated with epithelial-mesenchymal transition and stem-like properties in hepatocellular carcinoma.

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List of abbreviations: HCC: Hepatocellular Carcinoma; EMT: Epithelial Mesenchymal Transition; SMA: Smooth Muscle Actin; CSC: Cancer Stem Cells; TGF-β: Transforming Growth Factor beta.

Key words: Hepatocellular Carcinoma; Epithelial Mesenchymal Transition; gamma-Smooth Muscle Actin; Progenitors.

Conflict of interest: None

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Author contributions: NB, CM, CL, ADS and ClG: acquisition of data, analysis and interpretation of data. CD: statistical analysis. DS and CB: critical revision of the manuscript for important intellectual content. MFB and CaG: study concept and design, drafting of the manuscript, study supervision.

Abstract

Background and aims: The prognosis of hepatocellular carcinoma (HCC) is hampered by frequent tumour recurrence and metastases. Epithelial-Mesenchymal Transition (EMT) is now recognized as a key process in tumour invasion, metastasis and the generation of cancer initiating cells. The morphological identification of EMT in tumour samples from the expression of novel mesenchymal markers could provide relevant prognostic information and aid in understanding the metastatic process.

Methods: The expression of Smooth Muscle Actins was studied using immunofluorescence and immunohistochemistry assays in cultured liver cells during an induced EMT process and in liver specimens from adult and paediatric HCC series.

Results: We report here that in HCC cell lines treated with TGF-β or in HCC specimens, the expression of αSMA, a known mesenchymal marker of EMT, could never be detected. In addition, our *in vitro* studies identified the enteric form of SMA, γSMA, as being a marker of EMT. Moreover, this SMA isoform was expressed in 46% of 58 tumours from 42 adult HCC patients and in 90% of 16 tumours from 12 paediatric HCC patients. Interestingly, this expression was significantly correlated with poor tumour differentiation and progenitor cell features characterized by the expression of EpCAM and K19.

Conclusion: Taken together, our results support the conclusion that γSMA expression in HCC is strongly correlated with the EMT process, HCC aggressiveness and the identification of cancer stem cells. This correlation suggests that γSMA represents a novel and powerful marker to predict HCC progression.

Introduction

Hepatocellular carcinoma (HCC) is a major health problem in that it is the fifth most common cancer in the world and the third most frequent cause of cancer-related deaths. Most cases of HCC (80%) occur in livers that have become cirrhotic due to chronic Hepatitis B or C viral infection, alcohol abuse or obesity; all these conditions are characterized by long-standing hepatocyte damage and chronic inflammation leading to fibrosis [1]. Current chemotherapies are unable to exert a significant impact on patient survival. Although partial liver resection and liver transplantation have significantly improved survival with small tumours, the prognosis for HCC remains poor because of tumour invasiveness, frequent intrahepatic spread and extrahepatic metastases [2]. A clearer understanding of the molecular mechanisms underlying tumour invasiveness is therefore essential for the development of new therapies for HCC. It has been suggested that epithelial to mesenchymal transition (EMT) might be closely associated with the acquisition of aggressive traits by tumour cells, thus facilitating the early stages of metastasis and the subsequent dissemination of carcinoma cells [3] [4].

EMT is defined as a process during which epithelial cells lose their phenotypic characteristics and acquire mesenchymal cell features. Characteristic changes during EMT include the down-regulation of epithelial markers such as E-cadherin, and the up-regulation of mesenchymal markers such as vimentin and alpha smooth muscle actin (αSMA) [5]. More recently, EMT was linked to the emergence of cancer stem cells (CSC) [6] [7]. Indeed, it is now established that neoplastic epithelial cells re-enter the stem cell state through EMT. This has raised the intriguing possibility that the aggressiveness of carcinomas derives not only from the existing content of CSC but also from their proclivity to generate new CSC from non-CSC populations [8]. The correlation of carcinoma cell plasticity due to EMT with CSC properties may help to explain the role of CSC in the multistep progression of cancer. Indeed, oncogenic mutations that normally occur in differentiated cancer cells may involve CSC arising from EMT-

induced de-differentiation, and these CSC with new oncogenic mutations may then contribute to the progression of cancer towards metastasis. The emergence of these CSC may largely contribute to the resistance of cancers to chemotherapies.

The molecular mechanisms underlying EMT development have been studied extensively *in vitro*, and for liver cells, primary cultures of hepatocytes or hepatoma cell lines are able to develop EMT under stimuli such as TGF-β. Any morphological analysis of carcinoma by pathologists rarely recognizes the mesenchymal features of tumour cells on tissue slides. Indeed, EMT is a dynamic and continuous event that may be difficult to characterize in tumours because it only involves few cells at a given time. In the context of HCC, several studies have demonstrated a correlation between an up-regulation of EMT inducers such as transcription factors, Snail or Twist, and tumour invasiveness [9]. Such a correlation has also been established between the loss of E-cadherin, an epithelial marker, and poorly differentiated HCC [10]. The morphological identification of EMT in tumour samples from the expression of mesenchymal markers could offer a relevant prognostic marker and also help to understand the metastatic process. In order to identify on tissue sections the small minority of cells undergoing EMT among the tumour cell population, an immunohistochemical technique is the most appropriate approach. In addition, formalin fixed paraffin-embedded tissue samples are available for every tumour.

At present the best-identified mesenchymal marker of EMT in tumour cells is αSMA. The human genome contains six functional actin genes that are expressed in various muscle and non-muscle tissues [11]. Two smooth muscle actins have been described, αSMA which is encoded by the *ACTA2* gene and γSMA encoded by the *ACTG2* gene. Although they differ in their sequence by only three amino acids, different studies have described αSMA as the predominant variant in vascular and respiratory smooth muscle, with γSMA being the predominant isoform in smooth muscle cells of the gastrointestinal and urogenital tracts.

Because of their initially defined expression pattern, αSMA and γSMA are also referred as α-vascular and γ-enteric actins [12]. The distribution and role of γSMA is largely unknown.

We report here that the γSMA isoform could be considered as an EMT marker in HCC cell lines. Moreover, immunohistochemical analysis of a series of 58 tumours from adult patients and 16 tumours from paediatric patients revealed that γSMA, but not αSMA, was expressed in tumorous hepatocytes. The relevance of γSMA as an EMT marker for liver cells has been substantiated by *in vitro* data showing that this actin isoform becomes highly polymerized in hepatoma cell lines where EMT is stimulated by either TGF-β or over-expression of the Snail and Twist transcription factors. In view of the role of EMT in the emergence of CSC, the correlation between the expression of γSMA and progenitor markers (i.e. EpCAM or CK19) in HCC suggests that this actin isoform may represent a marker for non-CSC to CSC conversion. These inter-conversions are potentially important to the prognosis for HCC because CSC are more prone to dissemination and display resistance to anti-tumour therapies.

Materials and Methods

Vectors

The human cDNA clones pCMV6-Snail1 and pCMV6-Twist1 were obtained from Origen. The expression vector for pcDNA3.1_nV5 ACTG2 (V5-γSMA) was a kind gift from Dr Tuupanen.

Cells

The human hepatoma cell line HuH7, and the LX-2 human stellate cell line, were maintained in Dulbecco Modified medium containing 10% foetal calf serum (FCS). HuH7 cells were transfected with the different vectors using XtremeGENE HP DNA reagent (Roche).

Primary tumour hepatocytes were isolated from patients undergoing liver surgery for HCC, after obtaining their informed consent (Centre de Resources Biologiques (CRB) Paris Sud).

The tissue samples were washed in Williams medium, cut into 2 mm^3 fragments and incubated with Collagenase (Sigma Aldrich) (500 μg/ml, 2.4 mg/ml $CaCl_2$ in HEPES buffer, pH 7.4) at 37°C for 15 min. The cells were then washed twice and plated in Williams medium supplemented with 10% foetal calf serum. After 4 hours, the serum-containing medium was removed and the cells were cultured in Williams medium supplemented with 1 mg/ml bovine serum albumin, 100 μg/ml streptomycin and 100 U/ml penicillin, and treated with 2 ng/ml TGF-β for 48h. They were then processed for immunofluorescence.

Patients and tissue samples

Surgical liver specimens from 42 consecutive adult patients who underwent curative hepatic resection (n=19) or liver transplantation (n=23) for HCC at Hôpital Paul Brousse between January 1 and December 31, 2008, and 12 surgical specimens from paediatric patients who underwent hepatic resection (n= 5) or liver transplantation (n= 7) for HCC at Hôpital Bicêtre between 2006 and 2012, were collected after obtaining informed consent from the patients (CRB Paris Sud), and analysed for the purposes of the study. The 58 HCC nodules identified in the 42 liver surgical specimens from adult patients, and the 16 HCC identified in the 12 surgical specimens from the paediatric patients were reviewed by two pathologists. The diagnosis of HCC was confirmed for each nodule using standard morphological criteria and HepPar1 and/or the immunohistochemical expression of glypican. The following histopathological features were systematically assessed: size, tumour capsule, satellite nodules, percentage necrosis, differentiation according to Edmondson's grading, presence of cholangiolocellular and/or cholangiocellular component [13], macro and microvascular invasion.

Age, gender, underlying liver disease, type of surgery, and pre-and post-surgical treatments were recorded from the clinical charts.

As controls, 10 surgical liver specimens from normal livers, and 10 surgical liver specimens from cirrhotic livers were used to define γSMA staining in non-tumour liver tissues.

Antibodies

Antibodies directed against HepPar1, glypican3, EpCAM, cytokeratin 19 (K19), αSMA, (clone 1A4), vimentin (Clone V9) and E-cadherin were obtained from Dakocytomation, Glostrup, Denmark. Anti-γSMA antibodies (Clone E184) were obtained from Epitomics. Anti-Snail, anti-Twist and anti-V5 antibodies were sourced from Abcam. Anti-p38 antibody came from SantaCruz Biotechnology, and anti-calcitonin antibody was from Diagnostic Biosystems.

Western blotting

Cells were washed twice with PBS and lysed in RIPA buffer containing 0.5% SDS and Benzonase nuclease. Proteins were quantified with the Lowry assay and separated on SDS polyacrylamide gel, transferred on nitrocellulose membrane and blotted with different antibodies. Membranes were revealed using a chemioluminescence detection kit (ECL Plus, GE Healthcare) using a DCC camera (G Box Syngene).

Immunofluorescence staining

Cells were washed with PBS and fixed with a 4% PFA solution at 4°C for 20 min and permeabilized with PFS (saponin gelatin in PBS) for 30 min at 37°C. Cells were then incubated with primary anti-αSMA, γSMA or V5 antibody, and then with secondary Alexa Fluor 488 conjugated anti-mouse or Alexa Fluor 594 conjugated anti-rabbit antibody (Molecular Probes). They were then stained with Hoechst and examined by fluorescence microscopy.

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized and antigenic restoration was achieved by heating in citrate buffer pH6. Immunostaining was performed on these tissue sections using a Bond Max automate based on a labeled streptavidin-biotin (LSAB) method.

All immunohistochemical staining results were scored by two observers. Discrepant results were reexamined and assigned after concordant opinion. For combined tumors with cholangiolocellular and/or cholangiocellular component, only the HCC component was analyzed.

HepPar1, glypican3, K19 cytoplasmic staining, E-cadherin and EpCAM membranous staining were scored as a percentage of positive tumor cells. Moreover, the staining intensity of tumor cells for E-cadherin was assessed in comparison with the non-tumor adjacent hepatocyte staining as overexpressed, isoexpressed or underexpressed. αSMA and γSMA staining was defined as membranous and/or cytoplasmic and scored as a percentage of positive tumor cells. Negative controls were performed with a rabbit anti-calcitonin antibody. The threshold of positivity was defined as ≥10% for K19, EpCAM and γSMA.

Statistics

Detection of the γSMA histological marker enabled the distinction of two groups of patients (positive and negative for this marker). Odds ratios for each clinical and biological criterion were calculated for each group of patients (γSMA-positive and γSMA-negative). A meta-analysis performed on all the clinical and biological criteria taken into account during the study allowed testing of the heterogeneity of the calculated odds ratio. A forest plot was drawn using the odds ratios and 95% confidence intervals for each criterion. The significance of the meta-analysis was retained if the p-value of the heterogeneity test was lower than 0.05 and if the random effects on the forest plot were well centred [14].

Results

γSMA is expressed in hepatocarcinoma cells undergoing EMT

Using an anti-SMA antibody, we had previously reported that SMA was up-regulated in HuH7 cells undergoing EMT after treatment with TGF-β [15]. Using specific antibodies directed against alpha or gamma SMA, the present study focused on the expression and polymerization of these two actin isoforms in hepatic cells undergoing EMT following treatment with TGF-β. As shown in **Fig.1A**, immunofluorescence staining revealed a strong polymerization of γSMA in TGF-β–treated HuH7 cells in conditions where αSMA was not detectable. The same results were obtained when primary HCC cells were treated with TGF-β; suggesting that the gamma isoform of SMA might represent a mesenchymal marker of liver tumour cells. Hepatic LX-2 stellate cells were used as positive controls for αSMA expression, and interestingly the expression of both SMAs with strong co-labelling could be observed when these cells were activated by TGF-β. Furthermore, Western blot analysis demonstrated that αSMA was not detected in hepatic cells, whether they were or were not treated with TGF-β, while as expected, αSMA expression was easily detectable in LX-2 stellate cells after activation by TGF-β **(Fig. 1B. 1C)**.

The specificity of the anti-αSMA antibody used here is largely validated. However, few anti-γSMA antibodies are available. In order to verify that our anti γSMA antibody did indeed identify the γSMA isoform, we used a tagged-γSMA construct and studied the recognition of γSMA and αSMA antibodies in cells over-expressing the γSMA form. HuH7 cells were transfected with V5-tagged γSMA and analysed by immunofluorescence after 48h treatment with TGF-β. As shown in **Fig.1D**, a strict colocalisation was found when co-labelling with anti-V5 and anti-γSMA antibodies was performed, and strong polymerization in fibres was

observed. By contrast, no labelling was observed with αSMA antibodies despite the high homology between these isoforms. In line with this, Western blot analyses of cell extracts from HuH7 cells transfected or not with V5-γSMA showed that an over-expression of V5-γSMA was recognized by anti-V5 and anti-γSMA antibodies but not by anti-αSMA antibodies **(Fig.1E)**.

Although EMT is regulated by an elaborate interplay of signalling pathways, the full molecular reprogramming that occurs during EMT is mainly orchestrated by three major groups of transcription factors: the ZEB, Snail and Twist families. The exogenous over-expression of Twist1 increases the invasive and metastatic abilities of human cancer cells by promoting the down-regulation of E-cadherin and the induction of EMT. Likewise, the over-expression of Snail and Twist in hepatoma cell lines promotes EMT and the acquisition of an invasive phenotype [16]. Thus in order to gain further insight into the involvement of γSMA in EMT, we looked for γSMA polymerization in HuH7 cells over-expressing Twist1 and Snail1. For this purpose, HuH7 cells were transfected with a V5-tagged γSMA vector, with or without Twist and Snail vectors. Immunofluorescence staining was performed 48h after transfection. The polymerization of V5-tagged-γSMA was present in cells expressing Snail and Twist **(Fig.2A)**. This correlated with the findings of staining with phalloidin, a compound known to detect actin polymerization. No such polymerization of γSMA could be observed in cells expressing only V5-tagged γSMA **(Fig.2A)**. Western blotting analyses revealed the expression of Twist, Snail and V5 in the same experiment **(Fig.2B).**

Taken together, these results strongly suggest that γSMA is polymerized in hepatic cells having developed EMT induced by TGF-β or the over-expression of transcription factors such as Snail and Twist.

γSMA is expressed in human HCC

In vitro, EMT has emerged as a pivotal event in development of the invasive and metastatic potentials of cancer progression. However, although *in vivo* cancer cells undergo a de-differentiation process, EMT is still difficult to detect due to the tumour heterogeneity that is one of the hallmarks of HCC, and the transitory nature of EMT or EMT-like process. Our *in vitro* data prompted us to investigate whether γSMA expression might represent a marker for EMT that could be easily processed in tissue sections and used in routine clinical practice.

We first of all determined the expression of α and γSMA in ten normal livers. Both actin isoforms were expressed in the arteries, but their expression differed in other liver structures. Indeed, αSMA expression was mainly found in the non-parenchymal compartment. Interestingly, the canals of Hering were strongly labelled with γSMA antibodies and always negative with αSMA antibodies, suggesting that only the γSMA form is expressed in hepatic progenitor cells. The bile ducts were also positive for γSMA **(Fig.3A)**. Neither αSMA nor γSMA could be detected in hepatocytes, although a discrete labelling for γSMA was observed in the canaliculi **(Fig.3B)**.

The same results were obtained in ten cirrhotic livers with different aetiologies. γSMA was inconsistently expressed at the canalicular pole of hepatocytes. In HCC-affected cirrhotic livers, γSMA was not expressed in the cytoplasm of non-tumour hepatocytes, except focally in one case. Ductules within the fibrous bands were consistently positive, as were the bile ducts.

In the context of HCC, a total of 58 nodules corresponding to 42 patients were examined for γSMA expression. It was never possible to detect αSMA in tumour hepatocytes, whereas the expression of this actin form was easily detectable in the stromal compartment **(Fig.3C)**. By contrast, γSMA was detected in tumour hepatocytes from 46% of the tumours with membranous and/or cytoplasmic staining. γSMA expression was heterogeneous and

frequently observed at the invasive front of the tumour, but rarely throughout the nodule. In the panel of paediatric HCC samples, γSMA expression was observed in 15 out of 16 nodules, and here again its positivity was mainly observed at the edge of the nodules **(Fig.4A)**.

Meta-analyses of these 58 HCC nodules from adult patients indicated that γSMA expression was not correlated to age, type of surgery (liver transplantation or resection), tumour necrosis or neoadjuvant TACE treatment **(Table 1 and Fig. 5)**. By contrast, in the context of adult HCC, γSMA expression was significantly associated with a poor degree of tumour differentiation. In line with this finding, the majority of paediatric HCC cases were classified as Edmondson 3.

In order to correlate γSMA expression with a known marker of EMT, we studied the expression of E-cadherin, an epithelial marker which is lost during EMT. Although γSMA expression could be focally associated with a complete loss of E-cadherin expression **(Fig.3C)**, the extreme heterogeneity of E-cadherin staining within the same tumour hampered the demonstration of a firm correlation between a loss of E-cadherin and γSMA expression. However, in paediatric HCC, complete E-cadherin loss was observed in 13 out of 16 nodules, and E-cadherin expression was very weak in the others **(Table 2 and Fig. 4B)**. Thus a strong and significant association between γSMA expression and a loss of E-cadherin could be established, at least in the context of paediatric HCC.

Correlation between γSMA expression and stem cell markers

It is now well established that CSC can arise from tumour cells that have achieved EMT. Because of the strong expression of γSMA in the canals of Hering, we decided to determine whether a correlation exists between γSMA and the expression of stem cell markers in HCC. Interestingly, eight out of the nine HCC expressing CK19 were positive for γSMA staining. Furthermore, 10 out of the 12 HCC expressing EpCAM were also γSMA positive. Five CHC

co-expressed CK19 and EpCAM, and all of them were positive for γSMA expression. Meta-analyses of the data indicated that γSMA expression and progenitor markers were statistically correlated **(Table 1 and Fig. 5)**. These data therefore suggest that γSMA may be expressed in cancerous cells displaying the hallmarks of progenitor cells. It has been reported elsewhere that transarterial chemoembolisation (TACE) treatment may be associated with the emergence of CSC [17]. It is noteworthy that γSMA positivity was not correlated with previous TACE treatment.

With respect to paediatric HCC, a significant association was also observed between γSMA and EpCAM since 14 out of the 16 tumour nodules were positive for both proteins. In addition, the five nodules expressing CK19 were positive for γSMA staining **(Table 2)**. This result obtained in paediatric HCC therefore strongly support the hypothesis that γSMA may represent a marker of cancer stem cells or progenitor-like cells.

Discussion

Emerging data strongly suggest that like in other cancers, EMT may play an important role in tumour progression and the emergence of cancer stem cells in a context of hepatocellular carcinoma (HCC).

It has been shown that *in vitro* primary cultures of hepatocytes or hepatoma cell lines are able to develop EMT under stimuli such as TGF-β or the over-expression of Twist or Snail transcription factors [15] [18]. Our study demonstrated that TGF-β-induced EMT resulted in γSMA expression and organization in stress fibres in HuH7 cells. The use of a tagged-γSMA vector allowed us to show that the antibody used in this study recognized the γSMA isoform. Moreover, the over-expression of Snail and Twist, two transcription factors necessary for EMT progression, was sufficient to induce γSMA polymerization. These data strongly support the notion that γSMA represents an EMT marker in liver cancer cell lines.

To our knowledge, γSMA has never before been reported as an EMT marker. However, in many studies, phalloidin was used to detect polymerized actin during the EMT process, although it does not distinguish between different polymerized actins. It is therefore possible that γSMA expression is not restricted to hepatoma cells and may also be increased and polymerized in other cell lines undergoing EMT. In line with this, we observed that γSMA was the main polymerized SMA in MDCK cells after treatment with TGF-β (data not shown). Interestingly, it has been reported that TGF-β induced transcriptional activation of the γSMA gene. Indeed, increased steady-state levels of γSMA mRNA and the induced production and cytoskeletal polymerization of γSMA protein were observed during TGF-β-induced mesenchymal to myofibroblast differentiation [19].

Variants of γSMA have been implicated in familial visceral myopathy, a rare inheritable disease characterized by the impaired contraction of visceral smooth muscle cells and reduced bowel motility [20]. However, few data are available regarding a role for γSMA in cancer cells: It has been reported that *ACTG2* encoding for γSMA is one of the two most up-regulated genes in highly aggressive osteosarcoma cell lines when compared to non-aggressive cell lines [21]. Furthermore, γSMA mRNAs were shown to be up-regulated in cultured primary HCC cells, and particularly in cells with an aggressive phenotype [22]. These correlations between γSMA expression and cell aggressiveness or cellular motility are in agreement with our data and strongly support the notion that γSMA could be a marker for the invasive phenotype of cancer cells.

These *in vitro* results prompted us to look for γSMA expression in human HCC tissue sections in order to clarify the EMT process *in situ*. To the best of our knowledge, this is the first report of γSMA immunohistochemistry on human liver tissue. The positivity of the canals of Hering in normal livers, and of the ductular reaction in cirrhotic livers, strongly suggests that γSMA is a marker of liver progenitor cells. Normal or cirrhotic hepatocytes were negative,

indicating that γSMA, as previously described in several organs [12], is not involved in liver fibrosis. Immunohistochemistry on HCC in adult patients confirmed the expression of γSMA in 46% of the tumours with membranous and/or cytoplasmic staining of tumour cells, whereas αSMA was only expressed in stromal cells. These different intrahepatic localizations are in favour of complementary roles for these two SMA isoforms. The positivity of γSMA was only focal, and more frequently observed at the edge of the tumour. Strikingly, γSMA was expressed in the tumour cells of all but one of the paediatric HCC patients.

Until now, the *in situ* identification of EMT in HCC using mesenchymal markers was largely unsuccessful and produced contradictory results. It was mainly based on αSMA and vimentin immunostaining. Several papers have reported the expression of αSMA in HCC tissues, but only in cancer associated-fibroblasts and myofibroblasts of the stromal compartment and not in tumorous hepatocytes [23] [24] [25]. Similarly, systematic anti-αSMA immunostaining performed in a previously published study on a series of 86 HCC [26] was consistently negative in tumour cells (data not shown). However, in a recent paper, αSMA expression was detected at low levels by immunohistochemistry in HCC tumour cells [27]. Many anti-SMA antibodies recognize both α and γSMA, and because no precise indication was given concerning the anti-SMA antibodies used in that study, it is difficult to ascertain whether it was αSMA rather than γSMA that was actually expressed in these tumour cells.

The second mesenchymal marker widely employed to characterize EMT is vimentin. *In vitro*, vimentin expression in normal primary hepatocytes and HCC cell lines can be correlated with an EMT process [15]. However, in our cohort, we were never able to detect vimentin expression in HCC nodules. In a previous paper, we reported vimentin expression in intrahepatic cholangiocarcinoma using immunohistochemistry on tissue microarrays (TMA), whereas the 19 HCC included in these TMA were all negative [28]. Thus the lack of

vimentin detection in our present HCC cohort was not related to the antibodies used. Vimentin expression was also found to be consistently negative in another cohort of 86 HCC patients [26] (data not shown). In contradiction to our findings, vimentin expression in HCC tumour cells has been reported by different authors working on Asiatic cohorts [29] [30] [10]. These discordant results might be explained by the different aetiologies of European and Asian HCC. This discrepancy therefore warrants further investigation and might be clarified by comparing the expression of γSMA and vimentin in Asian cohorts.

As well as the over-expression of mesenchymal markers, EMT can also be characterized by a down-regulation of epithelial markers such as E-cadherin in HCC cells. However, in our study, E-cadherin staining appeared to be quite heterogeneous, even in non-tumour liver, so we were unable to demonstrate a statistically significant correlation between E-cadherin loss and γSMA expression at the level of whole tumours, even though this correlation could be observed focally. In paediatric HCC, E-cadherin was almost constantly down-regulated in association with γSMA expression.

Taken together, therefore, our results suggest that γSMA could be a robust and reliable *in situ* mesenchymal marker for an *in vivo* EMT process in the context of HCC.

γSMA expression in HCC was significantly associated with known histologically pronostic factors, such as poor tumour differentiation (Edmondson ≥ 3) and lack of tumour capsule formation. Moreover, although they did not meet the criteria for statistical significance, our data suggest that a correlation between γSMA expression and vascular invasion might also exist. Because most of the patients in our study benefited from liver transplantation, which totally modifies the natural history of HCC, it was not possible to determine a link between γSMA expression, tumour recurrence and overall survival.

Interestingly, γSMA expression in HCC tumour cells was strongly associated with stem/progenitor cell markers, namely CK19 and EpCAM. This result is in agreement with

γSMA expression in the canals of Hering and ductules, which are supposed to be the locations of adult liver progenitor cells. HCC with stemness-related marker expression is a recently proposed subtype of HCC in which a fraction of tumour cells (>5%) expresses stem/progenitor cell markers such as K19, CD133, c-kit, and EpCAM [31]. This subtype is associated with the expression of EMT markers and characterized by a poorer prognosis than HCC without stemness or progenitor markers.

Further evidence of the association between γSMA expression and stemness features was obtained by studying the paediatric HCC cohort. Indeed, these HCC differ from those in adults, with a nearly constant expression of stem/progenitor cell markers such as EpCAM, which we found was expressed in 14 out of the 16 tumours in our paediatric study. This result is in total agreement with a recent paper by Zen et al. who reported the expression of EpCAM in 25 out of 26 HCC nodules from a cohort of 12 children [32]. For these authors, EpCAM expression in childhood HCC may be attributable to the immaturity of neoplastic cells. In our series, the co-expression of γSMA and EpCAM, together with the down-regulation of E-cadherin, reinforced the paradigm that cancer stem-like cells might arise from cancer cells via an EMT process.

It would be of interest to examine the γSMA status of patients in the setting of other gastrointestinal cancers in order to determine whether expression of this protein is restricted to HCC or might represent a more general marker of CSC in endoderm-derived cancers.

Determining predictive biomarkers for HCC prognosis remains a challenge. The identification of γSMA as a potential mesenchymal marker of EMT, as well as EMT-induced stemness, constitutes an appreciable advance in predicting HCC progression and could be exploited for prognostic benefit.

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[32] Zen Y, Vara R, Portmann B, Hadzic N. Childhood hepatocellular carcinoma: a clinicopathological study of 12 cases with special reference to EpCAM. Histopathology 2013.

Fig.1

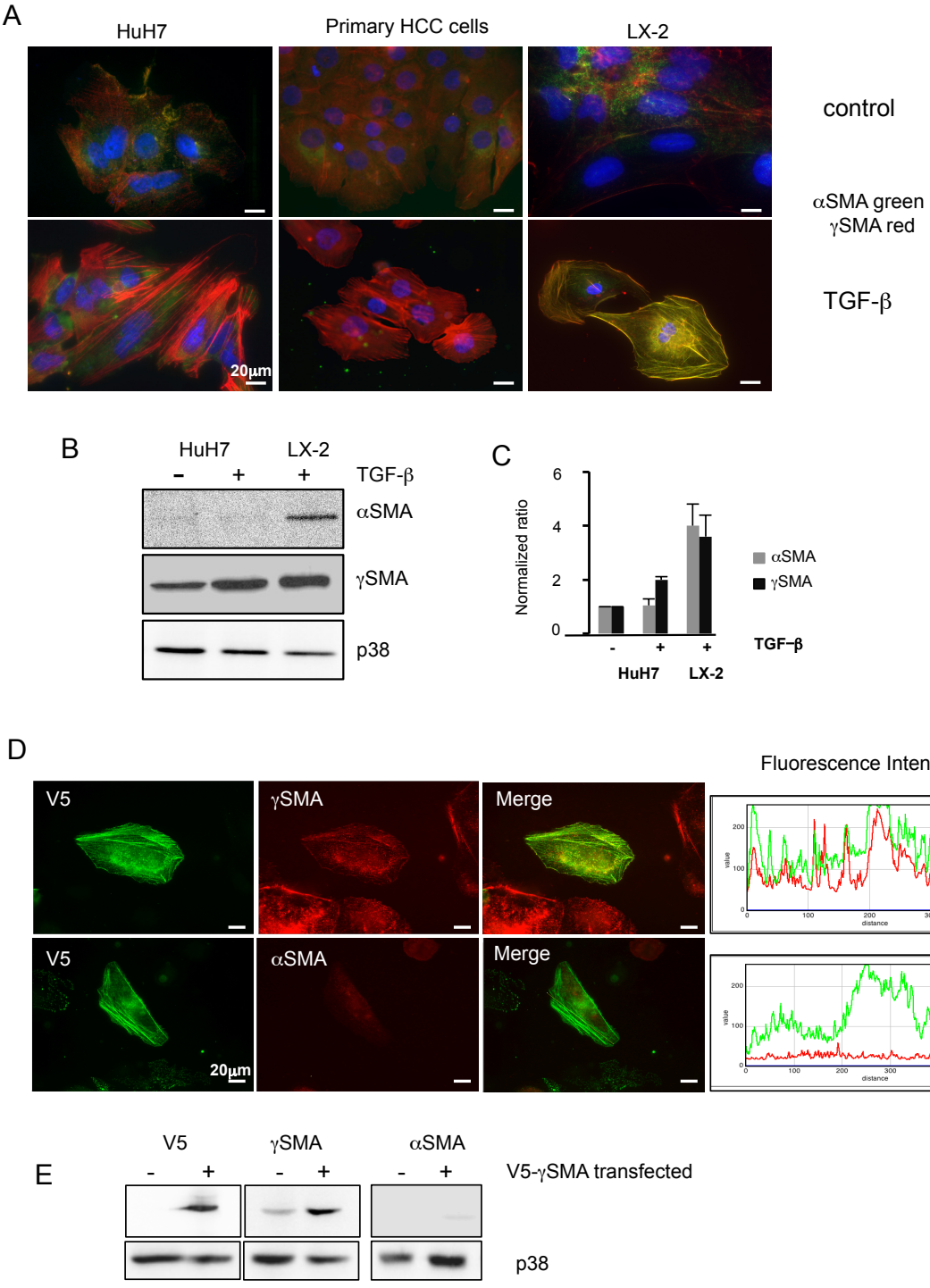

A
HuH7
Primary HCC cells
LX-2
control
αSMA green
γSMA red
TGF-β
20μm
B
HuH7
LX-2
- + + TGF-β
αSMA
γSMA
p38
C
Normalized ratio
6
4
2
0
αSMA
γSMA
- + + TGF-β
HuH7
LX-2
D
Fluorescence Intensity
V5
γSMA
Merge
V5
αSMA
Merge
20μm
value
distance
E
V5
γSMA
αSMA
- + - + - +
V5-γSMA transfected
p38

Fig.2

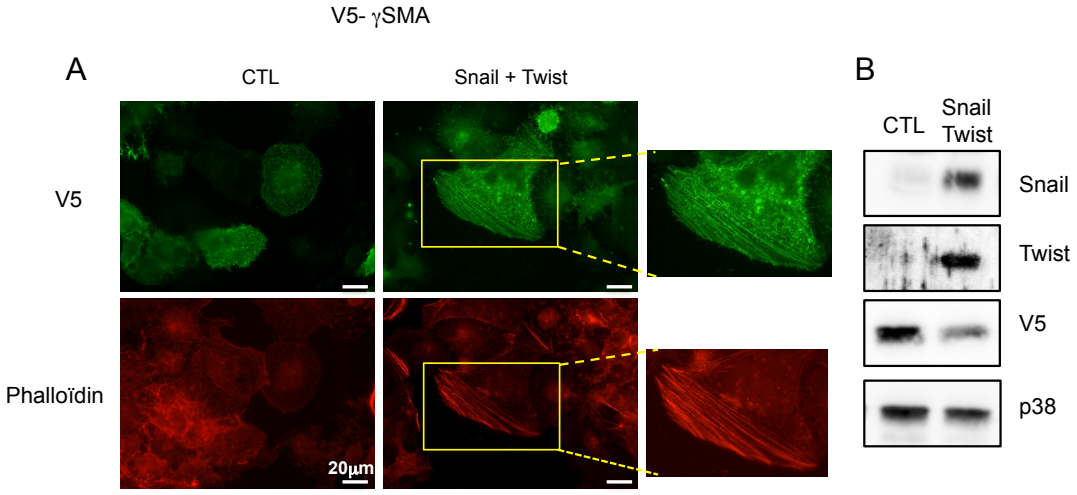

V5- γSMA
A
CTL
Snail + Twist
V5
Phalloïdin
20μm
B
CTL
Snail
Twist
Snail
Twist
V5
p38

Fig.3

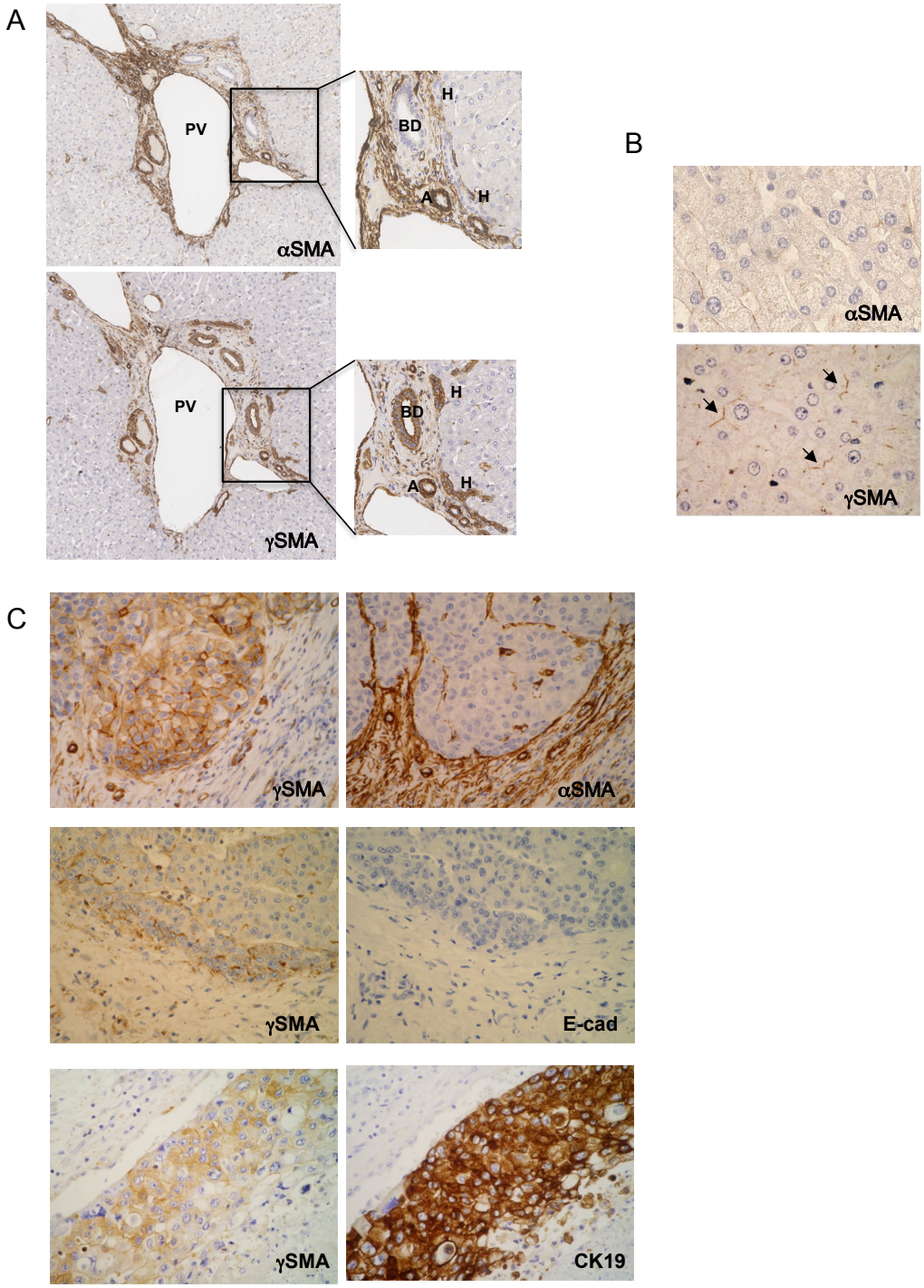

A
PV
αSMA
H
BD
A
H
PV
γSMA
H
BD
A
H
B
αSMA
γSMA
C
γSMA
αSMA
γSMA
E-cad
γSMA
CK19

Fig. 4

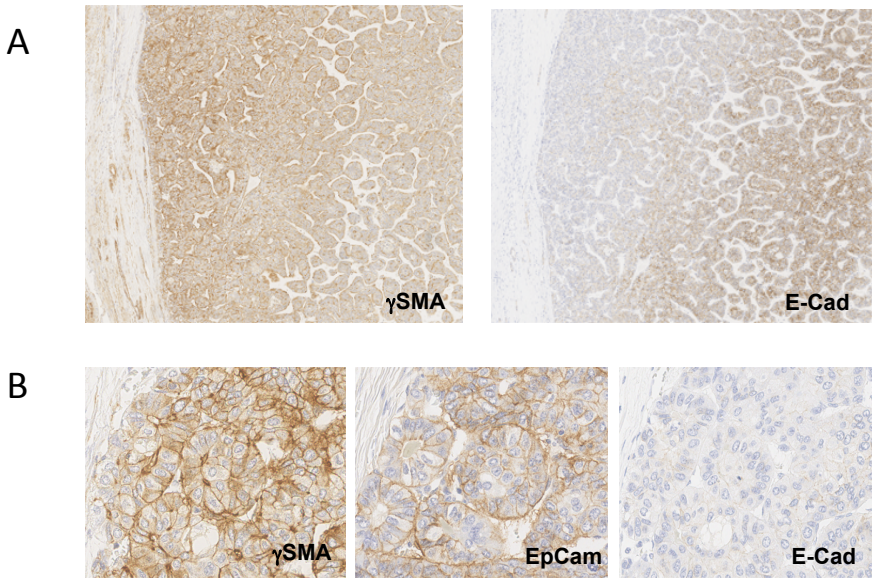


Fig.5

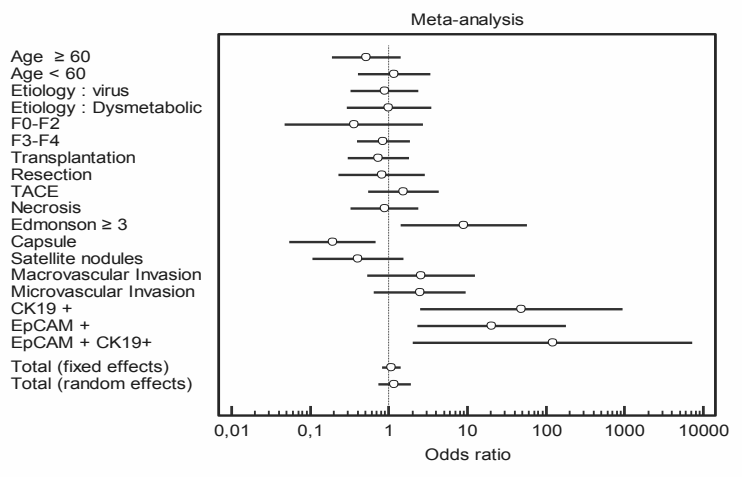

Meta-analysis
Age ≥ 60
Age < 60
Etiology : virus
Etiology : Dysmetabolic
F0-F2
F3-F4
Transplantation
Resection
TACE
Necrosis
Edmonson ≥ 3
Capsule
Satellite nodules
Macrovascular Invasion
Microvascular Invasion
CK19 +
EpCAM +
EpCAM + CK19+
Total (fixed effects)
Total (random effects)
0,01
0,1
1
10
100
1000
10000
Odds ratio

Table 1 : Correlation between γSMA expression, clinicopathological parameters and progenitor markers in HCC.

	Total	γSMA positive	Odds ratio	95% Confidence Interval
Total nodules	**58**	**27 (46.6%)**		
Age ≥ 60	31	13 (42%)	0.522	0.190 to 1.431
Age < 60	27	14 (51.8%)	1.16	0.399 to 3.373
Etiology :Viruses	31	15 (48.4%)	0.869	0.325 to 2.380
Etiology :Dysmetabolic	20	10 (50%)	1	0.289 to 3.454
F0-F2	8	3 (37.5%)	0.36	0.0476 to 2.725
F3-F4	50	24 (48%)	0.852	0.389 to 1.867
Transplantation	39	18 (46.2%)	0.735	0.302 to 1.790
Resection	19	9 (47.4%)	0.81	0.227 to 2.895
TACE	29	16 (55.2%)	1.515	0.538 to 4.264
Necrosis	31	15 (48.3%)	0.879	0.325 to 2.380
Edmonson ≥ 3	12	9 (75%)	9	1.418 to 57.119
Capsule	23	7 (30%)	0.191	0.0545 to 0.672
Satellite nodules	18	7 (38.9%)	0.405	0.106 to 1.547
Macrovascular Invasion	13	8 (61.5%)	2.56	0.527 to 12.431
Microvascular Invasion	18	11 (61.1%)	2.469	0.646 to 9.432
CK19 +	8	7 (87.5%)	49	2.531 to 948.671
EpCAM +	11	9 (81.8%)	20.25	2.319 to 176.800
EpCAM + CK19+	5	5 (100%)	121	2.017 to 7259.723

Table 2 : Correlation between γSMA, E-cadherin and progenitor marker expression in pediatric HCC.

	Total	γSMA positive	Odds ratio	95% Confidence Interval
Total nodules	**16**	**15 (93.7%)**		
E-cadherin negative	13	12 (92.3%)	144	8.043 to 2578.222
CK19 +	5	5 (100%)	121	2.017 to 7259.723
EpCAM +	14	14 (100%)	841	15.605 to 45324.593
EpCAM + CK19+	5	5 (100%)	121	2.017 to 7259.723

Legends to Figures

Fig.1. γSMA is expressed in TGF-β-induced EMT

(A) HuH7 cells and primary HCC tumour cells were treated with TGF-β for 48h and the expression of γSMA and αSMA was determined by immunofluorescence analysis. The images represent the merging of αSMA and γSMA staining. LX-2 stellate cells activated by TGF-β for 48h were used as a positive control for αSMA expression; one out of three representative experiments is shown. (B) HuH7 cells were treated with TGF-β for 48h and cell extracts were analysed by Western Blotting for the expression of αSMA and γSMA. TGF-β-treated LX-2 cells were used as positive controls for αSMA expression and p38 antibody was used as the control loading. (C) Densitometric analyses of the SMA/p38 ratio represent the mean +/- SD of three independent experiments.

(D) HuH7 cells were transfected with a V5-tagged vector coding for γSMA. Cells were treated with TGF-β for 48h, and the expression of V5, γSMA and αSMA was determined by immunofluorescence analysis. Expression levels of V5 and γSMA or V5 and αSMA were determined by measuring fluorescence intensity on a linear section of the cell. One representative experiment is shown. (E) HuH7 cells were transfected or not with a V5-tagged vector coding for γSMA and cell extracts were analysed for the expression of V5, αSMA and γSMA.

Fig.2. Snail and Twist expression induced γSMA polymerization

(A) HuH7 cells were transfected with a V5-tagged vector coding for γSMA alone (CTL) or together with vectors coding for Snail and Twist. The expression of V5 was determined by immunofluorescence analysis. Actin polymerization was revealed by Phalloidin staining. (B)

The expression of V5, γSMA, Snail and Twist was analysed by Western blotting. p38 was used as a control loading.

Fig.3. Immunochemical analyses of αSMA and γSMA expression in normal and HCC livers.

(A) Serial sections of normal livers were immunochemically stained with anti-αSMA or anti-γSMA antibodies. BD: bile duct, A: artery, H: canal of Hering, PV: Portal Vein. x50.

(B) Sections of normal livers were immunochemically stained with anti-αSMA or anti-γSMA antibodies. (➔ : canaliculi) x400

(C) HCC nodules were immunochemically stained with different antibodies. Serial sections stained with γSMA and αSMA; γSMA and E-Cadherin (E-cad); γSMA and CK19. x200

Fig.4: Serial sections of paediatric HCC samples were immunochemically stained with different antibodies. (A) γSMA, and E-cad. x50. (B) γSMA, E-cad and EpCAM. x400

Fig.5: Odds ratio meta-analyses of data from 58 HCC nodules.

Meta-analyses were performed on clinical and biological criteria.

Discussion

Les résultats acquis au cours de ce travail ont permis de mettre en évidence un nouveau rôle du TGF-β dans la pathogenèse des lésions liées au VHC. En particulier nos résultats démontrent qu'une protéine structurale du VHC, la protéine de capside provoque l'activation du TGF-β qui est sécrété sous une forme latente biologiquement inactive et module les effets biologiques de cette cytokine en augmentant ses effets pro-tumoraux.

I. Interaction de la protéine de capside avec la signalisation TGF-β

De nombreuses interactions entre le virus et les protéines cellulaires de l'hôte ont été décrites. Le VHC en interagissant avec plusieurs protéines cellulaires active ou inhibe différentes voies de signalisation et en conséquence module de nombreuses fonctions cellulaires. Des travaux réalisés au laboratoire ont montré l'existence d'une distribution différente des quasi-espèces codant pour la protéine de capside du VHC entre les zones tumorales et non tumorales du foie de patients infectés par le VHC et atteints de CHC, ouvrant la possibilité d'une sélection de souches ayant des propriétés fonctionnelles modifiées. Des expériences de pull down ont permis de mettre en évidence la liaison de la protéine Smad3 avec la protéine de capside provoquant une atténuation de l'activation de la voie canonique du TGF-β. De façon intéressante un variant de capside isolé à partir d'une zone tumorale (cT) lie Smad3 avec une affinité plus importante qu'un variant isolé d'un nodule cirrhotique (cNT) (Pavio et al, 2005). Dans ce travail, nous montrons que l'interaction de la protéine de capside avec la protéine Smad3 se traduit en une atténuation de la réponse cellulaire aux effets cytostatiques du TGF-β, en terme d'arrêt de croissance et d'induction d'apoptose; de façon remarquable, ces mêmes cellules résistantes au contrôle anti-tumoral du TGF-β, sont sensibles à la TEM induite par cette cytokine et répondent de manière soutenue, avec un changement morphologique vers le phénotype fibroblastique, et la synthèse de protéines telles que la SMA et la vimentine, qui sont des marqueurs caractéristiques de la TEM. Ces résultats sont en accord avec les travaux de Cheng et al, qui montrent que les protéines virales du HCV, capside et NS3 interagissent avec la protéine Smad3 et inhibent l'effet antiprolifératif du TGF-β (Cheng et al, 2004). De plus, dans les trois systèmes cellulaires analysés (lignée d'hépatome et hépatocytes primaires humains ou murins), nous avons observé que, lorsque le variant tumoral de la protéine de capside du VHC est exprimé, la synthèse des fibres d'actine est plus importante par rapport aux cellules contrôle et aux cellules exprimant le variant NT, renforçant notre hypothèse de départ d'une sélection de variants viraux ayant acquis de nouvelles capacités biologiques. Ces résultats suggèrent donc que la protéine de capside du VHC pourrait jouer un rôle important dans la carcinogenèse

hépatique, en déplaçant l'équilibre entre les effets anti-tumoraux et les effets pro-tumoraux de cette cytokine.

II. Mécanisme d'induction de la TEM par la protéine de capside

Il a longtemps été soutenu que des hépatocytes ne pouvaient subir une TEM. Dans notre étude, nous montrons que le TGF-β est capable d'induire une TEM dans des hépatocytes primaires humains ou murins surexprimant la protéine de capside. En particulier, nous avons noté que la protéine de capside est capable d'induire une TEM en absence de TGF-β exogène. Ce résultat est en adéquation avec les travaux de Dooley et al, qui témoignent que les hépatocytes peuvent subir une transition vers le phénotype mésenchymateux en réponse au TGF-β (Meindl-Beinker & Dooley, 2008). De plus, une étude récente désigne que la protéine de capside du VHC est capable d'induire une TEM et de promouvoir l'agressivité du CHC (Quan et al, 2013).

L'ensemble de ces observations suggère que les hépatocytes pourraient ainsi participer directement au processus de fibrogenèse.

Notre travail a permis de mettre en évidence l'importance de la voie de signalisation Smad3 dans l'induction de la TEM par le TGF-β. En effet, des cellules exprimant la protéine de capside mais déficientes pour la protéine Smad3 ne sont plus capables de répondre au TGF-β ni en terme cytostatique ni en terme de la TEM. En revanche une réintroduction d'une faible quantité de la protéine Smad3 dans ces cellules permet de restaurer l'effet pro tumoral de TGF-β. Ces résultats suggèrent qu'une activité transcriptionnelle diminuée de Smad3 suffit pour induire une TEM, alors qu'une activité transcriptionnelle plus élevée de Smad3 est nécessaire pour restaurer l'effet cytostatique du TGF-β dans ces cellules.

En accord avec nos résultats, des études témoignent de l'importance de la signalisation Smad3 dans le développement de la TEM *via* la phosphorylation de JNK (Alcorn et al, 2008). En effet, dans le cancer colorectal ou hépatique, l'activation de la voie JNK en phosphorylant Smad3 dans sa partie linker, attenue l'activité transcriptionnelle de Smad3 ce qui déplace les effets suppresseurs de tumeur du TGF-β en faveur de l'invasion cellulaire (Velden et al, 2011). De plus, une autre étude récente montre que des kératinocytes ayant une expression diminuée du récepteur de Type 1 du TGF-β répondent seulement aux effets pro-tumoraux du TGF-β, la diminution de l'expression du récepteur induit une réduction de 50% de la transcription des gènes dépendants des Smads (Kowli et al, 2013).

Nos résultats s'inscrivent parfaitement dans ce contexte, en démontrant par ailleurs qu'une TEM peut aussi se développer dans des hépatocytes humains.

Pour caractériser les mécanismes moléculaires impliqués dans les effets de la capside sur les réponses au TGF-β, nous avons recherché grâce à une étude transcriptomique les gènes dont l'expression est modulée par les protéines de capside dans des hépatocytes murins en culture primaire isolés à partir de souris transgéniques ou sauvages. De façon intéressante, nous montrons que 50% des gènes modulés par la protéine de capside en absence de TGF-β exogène sont des gènes cibles du TGF-β. Ce résultat suggère que la protéine de capside du VHC pourrait provoquer l'activation du TGF-β

En utilisant une méthode de dosage de la forme active du TGF-β reflétant une activité biologique, nous avons observé la présence de TGF-β actif dans des surnageants de cellules exprimant la protéine de capside. Cette observation a été confirmée dans des extraits de foies de souris transgéniques exprimant la capside cT ou cNT.

Plusieurs études ont montré une augmentation des taux de TGF-β chez les patients chroniquement infectés par le VHC (Divella et al, 2012). Toutefois, aucune étude ne précise si cette augmentation du TGF-β est corrélée avec une augmentation de la forme active du TGF-β. La distribution du TGF-β latent est ubiquitaire mais les réponses au TGF-β sont limitées par la disponibilité du TGF-β actif qui se produit localement et transitoirement en réponse à différents stimuli. L'activation du TGF-β est une étape importante dans les réponses biologiques du TGF-β.

Notre étude montre que le TGF-β activé lors de l'expression de la protéine de capside du VHC est capable de promouvoir la signalisation TGF-β des cellules du microenvironnement. En effet, des expériences de co-culture des hépatocytes exprimant la capside du VHC avec des cellules étoilées montrent que le TGF-β activé par les hépatocytes induit la différenciation des cellules étoilées vers un phénotype de type fibroblastique. Nos résultats sont en adéquation avec d'autres études qui témoignent de l'effet paracrine du TGF-β activé par les protéines virales. En effet, l'augmentation de l'expression du TGF-β dans des hépatocytes exprimant le réplicon du VHC est responsable de la différentiation des cellules étoilées et des effets pro-fibrosants (Schulze-Krebs et al, 2005). Une étude rapporte que des cellules étoilées hépatiques sont également activées par des hépatocytes exprimant les protéines du VHC (Presser et al, 2013). Des expériences *in vitro* montrent que des hépatocytes infectés par le VHC en co-culture avec des lymphocytes CD4 sont capables d'induire la différentiation des lymphocytes CD4 en Tregs, sous la dépendance du TGF-β (Hall et al, 2010). Néanmoins une autre étude implique la cytokine IL8 dans l'activation des cellules étoilées par des hépatocytes exprimant la protéine de capside (Clement et al, 2010).

Comme le TGF-β stimule la libération d'IL8 (Fong et al, 2008), il est possible que la cytokine IL8 soit augmentée dans des cellules exprimant la protéine de capside soit sous la dépendance du TGF-β.

L'ensemble de ces données est en faveur d'une activation du TGF-β par le VHC. Nos résultats désignent plus spécifiquement la protéine de capside comme étant responsable de cette activation. Par conséquent, elle contribue à la fibrogenèse hépatique en induisant une réponse pro-fibrotique des cellules étoilées du foie.

III. Mécanisme d'activation de la voie TGF-β par la protéine de capside

Une étude montre comment la protéine de capside active le promoteur du TGF-β via la voie de signalisation des MAP kinases et ainsi participe à la fibrose (Taniguchi et al, 2004). Notre analyse transcriptomique a permis d'identifier le gène *THBS1* comme étant surexprimé dans les hépatocytes murins transgéniques pour la protéine de capside. Ce gène code pour la thrombospondine-1 (TSP-1), une protéine sécrétée impliquée dans l'activation du TGF-β. Des expériences, utilisant un inhibiteur de TSP-1, ou des ARN interférant pour le gène TSP-1 sont en faveur de la participation de cette protéine dans l'activation du TGF-β latent médié par la protéine de capside. Nous montrons que le TGF-β est sécrété sous sa forme latente et que l'activation du TGF-β par la TSP-1 aurait lieu à l'extérieur de la cellule. Le travail de Presser et al, a mis en évidence que des hépatocytes infectés par le VHC induisaient également une expression et une activation du TGF-β par l'activité de la furine ou de la TSP-1 (Presser et al, 2011). D'autres mécanismes pourraient aussi être impliqués et plusieurs études révèlent que le VHC induit un stress oxydatif et une augmentation des ROS capables d'activer le TGF-β (Barcellos-Hoff & Dix, 1996; Okuda et al, 2002).

L'ensemble de nos travaux montre que les protéines de capside du VHC, cT et cNT sont capables d'activer le TGF-β dans des proportions comparables. Dans un hépatocyte, la protéine de capside et en particulier la protéine de capside isolée d'un variant tumoral module les réponses au TGF-β en favorisant la transition épithélium-mésenchyme.

De plus, notre étude identifie le TGF-β comme un acteur majeur des interactions entre le VHC et l'hôte et mettent en évidence un double rôle de la protéine de capside du VHC dans ces effets : la capside agit sur le microenvironnement par sa capacité à provoquer l'activation du TGF-β et module les réponses du TGF-β dans les hépatocytes en diminuant les effets anti-tumoraux et au contraire en augmentant ses effets pro-tumoraux (Fig.20).

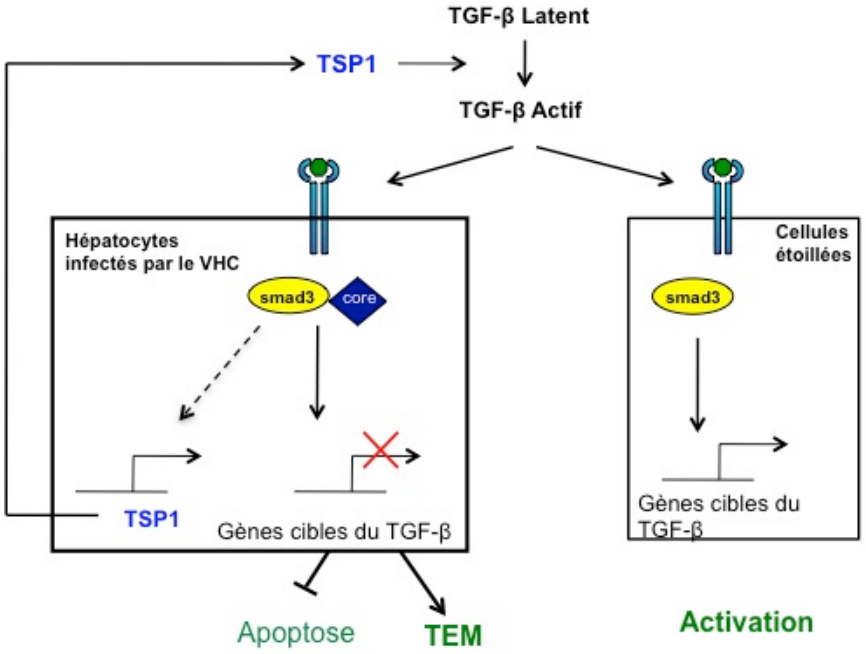


Figure 22 : Modèle d'implication du TGF-β dans la pathologie liée au VHC

La protéine de capside induit l'expression de la thrombospondine. Celle-ci active le TGF-β latent. Le TGF-β actif agit sur les hépatocytes infectés par le VHC, dans lesquels la protéine de capside interagit avec la protéine Smad3 et module les réponses biologiques du TGF-β en faveur de la promotion tumorale. De plus le TGF-β actif induit l'activation des cellules étoilées en myofibroblastes.

IV. Identification de la TEM dans le CHC

La TEM est un processus biologique qui permet le développement d'organes, la régénération de tissus, et l'invasion métastatique. L'identification de cellules subissant une TEM est difficile car ce processus est réversible et dynamique. La TEM est désormais considérée comme un acteur de la progression tumorale. Néanmoins, la réversibilité de ce processus rend difficile *in vivo* l'identification de cellules cancéreuses ayant développé un caractère mésenchymateux. En particulier, dans le CHC, très peu d'études ont porté sur l'identification de cellules mésenchymateuses dérivant d'hépatocytes cancéreux. Des travaux récents ont montré une corrélation entre l'expression des protéines impliquées dans la TEM, comme par exemple Snail, Twist ou vimentine, et le degré de malignité (grade de différenciation cellulaire et capacité d'invasion) (Giannelli et al, 2005; Kim et al, 2011).

Nos résultats sur l'activation du TGF-β nous ont incité à déterminer l'expression de marqueurs mésenchymateux dans le CHC. *In vitro*, la TEM est souvent associée à l'expression d'alpha Smooth muscle actin (αSMA). Il existe 2 isoformes de SMA dont la séquence ne diffère que de 3 acides aminés, l'αSMA, ou SMA aortique et la γSMA ou SMA entérique. Nous avons analysé in *vitro* par immunofluorescence la polymérisation de ces deux isoformes d'actine dans des hépatocytes ayant développé une TEM après traitement au

TGF-β ou par surexpression de la protéine de capside. En utilisant l'anticorps αSMA clone 1A4, aucune polymérisation d'actine n'est observée. Néanmoins avec l'anticorps γSMA clone E184, on observe une polymérisation de l'actine.

Nous avons donc analysé la spécificité de notre anticorps γSMA en transfectant des lignées d'hépatomes avec un plasmide étiqueté V5 codant pour le gène de la γSMA. Au cours de la TEM, l'expression de cette protéine est analysée avec nos deux anticorps SMA. L'expression et la polymérisation de la protéine exogène γSMA sont détectées seulement avec l'anticorps dédié et l'anticorps dirigé contre l'étiquette V5. De plus, nous avons également observé la polymérisation de cette actine dans un autre type cellulaire celui des MDCK (Madin-Darby canine Kidney). Ce résultat suggère que l'expression de la γSMA n'est pas restreinte aux hépatocytes.

Dans la littérature, très peu de données documentent la fonction de la γSMA. Des mutations des variants du gène γSMA sont responsables de maladies rares comme la myopathie viscérale familiale et le syndrome de Berdon caractérisées par un dysfonctionnement des muscles lisses entériques (Lehtonen et al, 2012) ;(Thorson et al, 2014).

Cependant, en accord avec notre résultat, une étude montre que le TGF-β régule l'activité transcriptionnelle et la production de la protéine γSMA (Hirschi et al, 2002). De plus, une corrélation a été établie entre l'expression de la γSMA et la capacité invasive des cellules primaires issues de CHC (Lin & Chuang, 2012). Dans un autre cancer, l'ostéosarcome, une surexpression de γSMA a aussi été observée (Li et al, 2010) ;(Lauvrak et al, 2013).

L'ensemble de ces études concernant l'expression de la γSMA est en accord avec nos résultats obtenus *in vitro*, suggérant que la γSMA puisse être un marqueur de cellules cancéreuses ayant un phénotype mésenchymateux.

Afin d'analyser l'expression de la γSMA *in vivo*, nous avons mené une étude à partir d'une cohorte de patients atteints de CHC d'étiologies différentes.

Dans le foie normal, l'expression de l'αSMA et de la γSMA est différente : bien que l'expression des deux formes de SMA soit observée dans les artères, l'αSMA est localisée dans le compartiment non parenchymateux alors que l'expression de γSMA est située au niveau des canalicules biliaires et dans les canaux de Hering. De ce fait, l'expression de la γSMA pourrait être associée à des propriétés de cellules progénitrices.

L'analyse immunohistochimique portant sur 58 nodules tumoraux montre montre l'absence d'expression de l'αSMA dans les hépatocytes tumoraux. De même, dans une autre étude réalisée au sein de l'unité, sur des CHC de patients co-infectés soit par le VHB/VIH soit par le VHC/VIH, l'immunomarquage pour l'αSMA dans les hépatocytes tumoraux s'est toujours révélée négative (Vibert et al, 2011). Plusieurs travaux ont rapporté l'expression de l'αSMA dans le CHC. Dans trois études, l'expression de αSMA était limitée aux fibroblastes et myofibroblastes présents dans le stroma et jamais dans les hépatocytes tumoraux (Takamura et al, 2013); (Yang et al, 2013) ;(Parikh et al, 2014). En revanche, un travail a mis en évidence l'expression de l'αSMA par immunohistochimie dans 29,4 % de cellules tumorales (Li et al, 2013). Ce résultat différent des nôtres, peut être expliqué par la spécificité de l'anticorps utilisé qui peut reconnaître les deux isoformes alpha et gamma SMA. Cette étude révèle aussi une augmentation de l'ARN messagers de la protéine αSMA dans les CHC. Ce résultat cependant peut être le reflet de l'expression de l'αSMA des cellules du stroma et en particulier celui des cellules étoilées. Par conséquent, il est difficile d'affirmer s'il s'agit d'une augmentation de la transcription de la protéine αSMA dans des hépatocytes.

La γSMA est par contre exprimée dans les hépatocytes tumoraux de 46% de tumeurs de notre série avec une expression membranaire et/ou cytoplasmique.

Le deuxième marqueur mésenchymateux largement utilisé pour caractériser la TEM est la vimentine**.** En effet, lors de la TEM *in vitro*, nous avons observé l'expression de la vimentine dans les hépatocytes primaires et les lignées cellulaires exprimant la protéine de capside (Battaglia et al, 2009). Néanmoins, dans notre cohorte de patients atteints de CHC l'expression de la vimentine n'a jamais été détectée dans les tumeurs alors que le même anticorps détecte cette protéine dans des cholangiocarcinomes (Dos Santos et al, 2010). À l'inverse de nos résultats, plusieurs études à partir d'une cohorte asiatique ont révélé une expression de la vimentine au sein de cellules tumorales du CHC. Ces résultats discordants peuvent s'expliquer par l'origine différente des patients de ces cohortes, l'une européenne et les autres asiatiques.

La TEM est aussi caractérisée par la dérégulation des marqueurs épithéliaux comme la E-cadhérine. L'expression de la E-cadhérine dans notre cohorte est assez hétérogène et nous n'avons pas réussi à mettre en évidence une corrélation statistiquement significative entre la perte de la E-cadhérine et l'expression de la γSMA au niveau de la totalité de la tumeur. Cependant, localement, une corrélation entre l'expression de γSMA et la perte d'expression de la E-Cadhérine a été observée. L'analyse histologique met en évidence que

dans cette cohorte l'expression de la γSMA est aussi associée à l'agressivité des tumeurs car elle est corrèlée avec l'absence de capsule, l'invasion vasculaire et un grade d'Edmondson 3, qui signifie une faible différentiation des hépatocytes.

Il reste difficile d'établir une corrélation entre l'expression de la γSMA et le suivi longitudinal des patients car la plupart des patients de la cohorte ont bénéficié d'une transplantation hépatique, ce qui modifie l'histoire naturelle de la maladie.

De façon intéressante, nous montrons qu'il existe une corrélation significative entre l'expression de γSMA et l'expression de marqueurs progéniteurs comme EpCam ou CK19, suggérant que l'expression de γSMA peut refléter un caractère de cellule souche cancéreuse. Afin de conforter cette hypothèse, nous avons étudié l'expression de la γSMA dans une cohorte de CHC pédiatriques. Une étude récemment publiée montre que l'expression de EpCAM est observée dans 25 nodules de CHC sur 26 d'une cohorte de 12 enfants (Zen et al, 2013). Les auteurs de cette étude associent l'expression de EpCam à l'état immature des cellules néoplasiques. Dans notre cohorte, et en accord avec le travail de Zen et al. 14 nodules sur 16 étudiés expriment EpCam. Ils expriment tous la γSMA. De plus, les 5 nodules positifs pour CK19 sont aussi positifs pour la γSMA. Ce résultat est fortement en accord avec une étroite corrélation entre l'expression de γSMA et l'expression de marqueurs progéniteurs.

De plus, dans ces CHC pédiatriques, 12 tumeurs sur 13 présentent une perte de l'expression de la E-cadhérine associée à l'expression de la γSMA suggérant le développement d'une TEM.

L'ensemble de ces observations renforce l'idée que les cellules souches cancéreuses pourraient provenir de cellules cancéreuses subissant une TEM.

Ce travail a permis l'identification de la γSMA en tant que marqueur potentiel de cellules subissant une TEM dans le CHC ainsi que de l'état progéniteur induit par la TEM. La détection de la γSMA pourrait donc constituer un progrès appréciable dans la prédiction de la progression du CHC et être exploitée à visée pronostique.

Conclusions et perspectives

Ce travail a permis de mettre en évidence un nouveau rôle de la protéine de capside du VHC dans la fibrogenèse et la carcinogenèse hépatiques.. En effet, nous avons décrit que la protéine de capside jouait un double rôle en modifiant d'une part,

-les réponses biologiques du TGF-β

- en conférant une résistance aux effets cytostatiques
- en favorisant l'effet pro-tumoral de celui-ci, facilitant ainsi l'invasion cellulaire;

D'autre part,

-la protéine de capside active le TGF-β latent provoquant ainsi la signalisation TGF-β des hépatocytes infectés et des cellules stromales du foie.

En conformité avec ces observations, l'analyse transcriptomique a révélé que la protéine de capside des hépatocytes murins infectés module des gènes parmi lesquels 50% sont des gènes cibles du TGF-β.

Nos résultats suggèrent donc que la protéine de capside du VHC pourrait avoir un potentiel oncogène par ses effets sur l'activation et les réponses biologiques du TGF-β.

Pour mieux comprendre les mécanismes moléculaires associés à la survenue et à la progression du carcinome hépatocellulaire lié au VHC et en particulier le rôle du TGF-β dans cette progression cancéreuse, notre objectif est de déterminer le phosphoprotéome de cellules exprimant la capside du VHC pour évaluer de façon globale l'activation des différentes voies de signalisation modulées par cette protéine et leur relation avec la signalisation du TGF-β.

La suite du projet consistera à étudier *in vivo* le rôle de la protéine de capside du VHC dans la cancérogénèse hépatique. Différents travaux portant sur des souris transgéniques pour la capside du VHC sont contradictoires. Une seule étude a montré que ces souris pouvaient développer un CHC, toutes les autres ont montré que ces souris ne développaient pas de pathologie particulière à part une stéatose dans certains cas.

Nous avons établi des lignées de souris transgéniques pour la capside cT ou cNT. Ces souris, et en particulier les souris cT développent une stéatose à l'âge de 9 mois, mais ne présentent aucun CHC sur un suivi de 24 mois. Dans le but de rechercher si la protéine de capside peut accélérer la survenue d'un CHC, nous avons croisé ces souris avec des souris

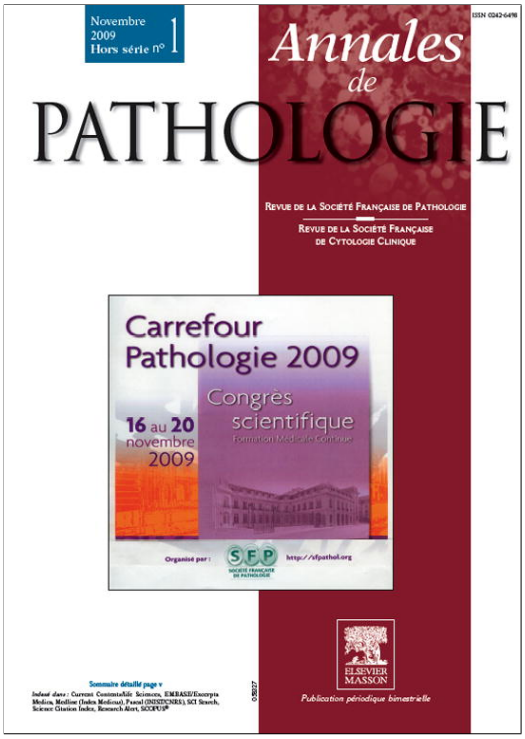
transgéniques pour l'oncogène c-myc qui développent un CHC. Des résultats préliminaires indiquent que l'expression de la capside chez ces souris double transgéniques accélère le développement des tumeurs. A partir de l'analyse du phosphoprotéome, l'objectif est maintenant de rechercher les voies de signalisation activées par la capside et impliquées dans le développement des tumeurs

Annexes

Annexe 1

Transition Épithélio-Mésenchymateuse et Carcinome Hépatocellulaire

Sérena Battaglia, Nassima Benzoubir, Maria-Rosa Ghigna, Catherine Guettier, Christian Bréchot, Marie-Françoise Bourgeade. Annales dePathologie 2009



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SYMPOSIUM / *Transition épithélio-mésenchymateuse en cancérologie*

Transition épithélio-mésenchymateuse et carcinome hépatocellulaire☆

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Dans le foie, l'activation des cellules étoilées en myofibroblastes a longtemps été considérée comme la principale source de cellules produisant les protéines de la matrice extracellulaire. Néanmoins, plusieurs études ont clairement démontré que des hépatocytes adultes pouvaient développer une transition épithélio-mésenchymateuse et participer ainsi au développement de la fibrose [1,2].

La transition épithélio-mésenchymateuse est un processus physiologique qui intervient au cours du développement embryonnaire et qui pourrait aussi intervenir dans l'invasion tumorale. D'une manière générale, au cours de la transition épithélio-mésenchymateuse, les cellules, sous l'influence de différents facteurs perdent leurs marqueurs épithéliaux comme la E-cadhérine, responsable des jonctions cellulaires et acquièrent des marqueurs mésenchymateux comme l'actine musculaire lisse ou la vimentine. Cette transition permet aux cellules d'augmenter leur capacité migratoire. Différents travaux ont montré que l'expression de plusieurs facteurs de transcription tels que Snail, Twist, Zeb1 et Zeb2 étaient nécessaires pour réprimer l'expression de la E-cadhérine qui représente la première étape de la transition épithélio-mésenchymateuse [3]. Plus récemment, il a été montré que l'expression de Twist était augmentée dans le carcinome hépatocellulaire [4] suggérant que des hépatocytes tumoraux pourraient aussi développer une transition épithélio-mésenchymateuse.

La transition épithélio-mésenchymateuse peut être induite in vitro par différentes cytokines et en particulier le *Transforming Growth Factor beta* (TGF-bêta) qui augmente l'expression des facteurs Snail et Twist. Le TGF-bêta est une cytokine multifonctionnelle qui a un rôle ambivalent dans la tumorigenèse, en inhibant d'une part, la croissance tumorale et en provoquant l'apoptose dans les stades précoces et en favorisant, d'autre part, le développement tumoral et l'invasion métastatique dans les stades tardifs [5].

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Différents facteurs modulant la signalisation du TGF-bêta ont été impliqués dans la progression tumorale et en particulier certains virus oncogènes. En étudiant le rôle du virus de l'hépatite C au cours de la carcinogenèse hépatique, nous avons identifié une interaction entre la protéine de capside de ce virus et la signalisation du TGF-bêta. Cette interaction se traduit par une augmentation des effets protumoraux du TGF-bêta. En effet, l'expression de la protéine de capside du virus de l'hépatite C (VHC) atténue les réponses au TGF-bêta en termes d'arrêt de croissance ou d'apoptose et au contraire favorise le développement d'une transition épithélio-mésenchymateuse dans des lignées d'hépatomes humains ainsi que dans des hépatocytes en culture primaire. De façon intéressante, nous montrons que la protéine de capside du virus de l'hépatite C induit l'expression de marqueurs de transition épithélio-mésenchymateuse comme la vimentine ou l'actine musculaire lisse même en absence de TGF-bêta exogène. Cet effet est inhibé en présence d'un inhibiteur du récepteur du TGF-bêta suggérant que l'expression de cette protéine virale provoque une activation du TGF-bêta endogène.

Ces résultats suggèrent un rôle direct de la protéine de capside du virus de l'hépatite C dans la progression tumorale des hépatocytes à travers ses effets sur les réponses biologiques du TGF-bêta. Ainsi, le virus de l'hépatite C, à travers la protéine de capside, pourrait favoriser la transition épithélio-mésenchymateuse, stimulant ainsi le processus de dissémination tumorale. Ces mécanismes sont actuellement évalués à partir de carcinomes hépatocellulaires humains développés sur cirrhose C [6].

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Annexe 2

« L'activation de la voie p38-MAPK par FLT3 participe au contrôle de la mégacaryopoïèse dans la myélofibrose primitive. »

FLT3-mediated p38-MAPK activation participates in the control of megakaryopoiesis in primary myelofibrosis. Desterke C, Bilhou-Nabéra C, Guerton B, Martinaud C, Tonetti C, Clay D, Guglielmelli P, Vannucchi A, Bordessoule D, Hasselbalch H, Dupriez B, **Benzoubir N**, Bourgeade MF, Pierre-Louis O, Lazar V, Vainchenker W, Bennaceur-Griscelli A, Gisslinger H, Giraudier S, Le Bousse-Kerdilès MC; French Intergroup of Myeloproliferative Disorders,; French INSERM; European EUMNET Networks on Myelofibrosis. Cancer Res. 2011 Apr 15;71(8):2901-15. doi: 10.1158/0008-5472.CAN-10-1731. Epub 2011 Apr 12. Erratum in: Cancer Res. 2011 Jun 1;71(11):4049-50.

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Cancer Research

FLT3-Mediated p38–MAPK Activation Participates in the Control of Megakaryopoiesis in Primary Myelofibrosis

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Molecular and Cellular Pathobiology

Cancer Research

FLT3-Mediated p38–MAPK Activation Participates in the Control of Megakaryopoiesis in Primary Myelofibrosis

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Abstract

Primary myelofibrosis (PMF) is characterized by increased number of hematopoietic progenitors and a dysmegakaryopoiesis which supports the stromal reaction defining this disease. We showed that increased ligand (FL) levels in plasma, hematopoietic progenitors, and stromal cells from PMF patients were associated with upregulation of the cognate Flt3 receptor on megakaryocytic (MK) cells. This connection prompted us to study a functional role for the FL/Flt3 couple in PMF dysmegakaryopoiesis, as a route to reveal insights into pathobiology and therapy in this disease. Analysis of PMF $CD34^+$ and MK cell transcriptomes revealed deregulation of the mitogen-activated protein kinase (MAPK) pathway along with Flt3 expression. In PMF patients, a higher proportion of circulating $Flt3^+CD34^+CD41^+$ cells exhibited an increased MAPK effector phosphorylation independently of $Jak2^{V617F}$ mutation. Activation of FL/Flt3 axis in PMF MK cell cultures, in response to FL, induced activation of the p38–MAPK cascade, which is known to be involved in inflammation, also increasing expression of its target genes (*NFATC4*, *p53*, *AP-1*, *IL-8*). Inhibiting Flt3 or MAPK or especially p38 by chemical, antibody, or silencing strategies restored megakaryopoiesis and reduced phosphorylation of Flt3 and p38 pathway effectors, confirming the involvement of Flt3 in PMF dysmegakaryopoiesis via p38 activation. In addition, in contrast to healthy donors, MK cells derived from PMF $CD34^+$ cells exhibited an FL-induced migration that could be reversed by p38 inhibition. Taken together, our results implicate the FL/Flt3 ligand–receptor complex in PMF dysmegakaryopoiesis through persistent p38–MAPK activation, with implications for therapeutic prospects to correct altered megakaryopoiesis in an inflammatory context. *Cancer Res; 71(8); 2901–15. ©2011 AACR.*

Introduction

Primary myelofibrosis (PMF) is a Ph (Philadelphia)-negative myeloproliferative neoplasm (MPN) characterized by extramedullary hematopoiesis with splenomegaly, myelofibrosis, and neoangiogenesis. The clonal myeloproliferative process is illustrated by an increased number and mobilization of hematopoietic stem cells/progenitors (HSC/HP) with a hypersensitivity to cytokines partly resulting from $Jak2^{V617F}$ or $MPL^{515L/K}$ mutations (1–6). A prominent proliferation of megakaryocytes (MK) with a dysmegakaryopoiesis characterized by dysplastic MK with plump lobulation of nuclei and disturbance of nuclear/cytoplasmic maturation is observed in patients (7). Previous studies (8–10) have suggested that bone marrow fibrosis was secondary to fibroblast activation by inflammatory and fibrogenic growth factors including TGF-β produced by the necrotic and dysplastic MKs (11, 12). Recently, evidences have been accumulating that stromal cells also contribute to the hematopoietic clone development through specific and mutually dependent interactions with pathologic HSCs (13).

FL, the ligand for the tyrosine kinase receptor Flt3 (14), is mainly expressed in stromal cells, including fibroblasts, likely stimulated by TGF-β. FL is of paramount importance in the proliferation of primitive hematopoietic progenitors (15–17) as confirmed by the reduced myeloid and B-lymphoid progenitor content observed in $FL^{-/-}$ and $Flt3^{-/-}$ mice (18, 19).

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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However, role of FL on normal megakaryopoiesis appears controversial because it does not stimulate, as a single agent, MK progenitors (20), but it enhances their proliferation in association with cytokines such as thrombopoietin (TPO; ref. 21). Its cognate receptor, Flt3, is expressed by HSC and myeloid progenitors (22) in which it signals through several downstream pathways including the mitogen-activated protein kinase (MAPK) pathway (16). Alterations of Flt3 are frequently observed in leukemic cells and mutations have been detected in about 30% of patients with acute myelogenous leukemia as well as in patients with acute lymphocytic leukemia or myelodysplastic syndrome. Mutations most often involve small tandem duplications within the juxtamembrane domain of the receptor (Flt3-ITD), resulting in constitutive tyrosine kinase activity. The recent demonstration that expression of Flt3-ITD in murine marrow cells results in a lethal MPN (23) strengthens the role of FL/Flt3 deregulation in leukemia and encourages us to investigate its possible contribution to human MPN pathogenesis and especially in PMF, in which a deregulation of primitive hematopoieisis is strongly associated with profound alterations of stroma.

In this study, we evidenced that the FL plasma level is increased in PMF patients and is overexpressed by stromal and $CD34^+$ hematopoietic cells. FL augmentation is associated with an aberrant Flt3 expression in $CD34^+$ and MK cells and with an alteration of the MAPK pathway and especially of p38, in patients, independently of their Jak2 mutational status. We further showed that the persistence of Flt3 signaling, which elicits activation of MAPK, known to be involved in MK polyploidization (24), participates in the dysmegakaryopoiesis that characterizes PMF. Taken together, our results implicate the "FL/Flt3" couple in PMF MK deregulation through persistent p38–MAPK activation.

Materials and Methods

Patients

One hundred twenty-six PMF patients [$Jak2^{V617F}$ ($n = 51$), $Jak2^{WT}$ ($n = 45$), and Jak2 status not determined ($n = 30$)], obtained from clinicians of the French and European networks, and 90 unmobilized healthy donors (HD) were enrolled. Samples were obtained with the informed consent of subjects according to the Helsinki declaration.

Cell selection

$CD34^+$ cell selection was carried out on mononuclear cells (MNC) from peripheral blood (PB) or bone marrow (BM) samples from PMF patients and HD as previously described (purity > 97%; ref. 25). Stromal cells obtained from osteomedullar biopsies or hip surgery from PMF patients and HD, respectively, were cultivated for 3 to 4 passages in DMEM (Dulbecco's modified Eagle's medium) + 10% FCS (fetal calf serum). FL plasma level was quantified using Quantikine ELISA (R&D Systems).

Microarray analysis and quantitative RT-PCR

For microarray technique, see Supplementary Material. For quantitative reverse transcription PCR (QRT-PCR), total RNAs were subjected to RNase-free DNase and converted into cDNA by using the Reverse Transcription Kit (Applied Biosystem). cDNA (2 μL) was added to the QuantiTect SYBR Green amplification reaction (QIAGEN) in a 20 μL final volume and 10 pmol of each primer (Supplementary Table S1) were added to carry out specific amplification. *RPL38* was used as housekeeping gene and relative quantification was based on the $2^{\Delta\Delta CT}$ method (26).

Phenotypic analysis of $CD34^+$ cells

Cells (5×10^4) were labeled with 2 μg/mL of the following monoclonal antibodies (mAb): CD38-fluorescein isothiocyanate (FITC; clone-T16) or CD41-FITC (clone-P2; Beckman Coulter) versus IgG1-FITC isotype; Flt3-PE (CD135, clone-4G8; BD Pharmingen) or CD41-PE (clone-5B12; Dako) versus IgG1-PE isotype; CD34-PerCP (clone-8G12) versus IgG1-PerCP isotype; CD41-APC (clone-386629) versus IgG1-APC isotype (BD Pharmingen). Membrane antigen fluorescence was quantified by using CellQuest software on a FACScalibur (Becton Dickinson). Live cells (5×10^3) were analyzed.

MK derived from $CD34^+$ cultures

$CD34^+$ cells (5×10^4/500 μL per well) were cultured for 10 to 14 days in MK differentiation medium [SYN.H serum-free medium containing Recombinant human stem cell factor (rhSCF): 5 ng/mL; rhIL-3: 2 ng/mL; rhIL-6: 1 ng/mL; rhIL-11: 40 ng/mL, rhTpo: 50 ng/mL; AbCys Synergie] with or without inhibitors and viability was evaluated by trypan blue. For RNA silencing, cells were cultured for 6 days and distributed in 24 well per plate per 250 μL with or without control or specific siRNA (1 μg) and a vector MISSION II (1/50; Sigma). Biological effect of siRNA was evaluated after 48-hour incubation.

Megakaryocyte ploidy measurement

DNA content was measured by incorporation of propidium iodine (PI). Megakaryocytes obtained at day 12 (D12) of culture were fixed with 70% ethanol (−20°C), centrifuged and treated with RNase (500 μg/mL) and PI (50 μg/mL; Sigma). Live cells (3×10^4) were analyzed and the percentage of polyploid cells (8N-256N) was determined on FACScalibur with CellQuest software. The B/S ratio (big/small MK proportion) was calculated as following: $\Sigma(64N + 128N + 256N)/\Sigma(8N + 16N + 32N) \times 100$.

For cyclin D3 expression, MK derived from PMF $CD34^+$ culture (D10) were labeled with a cyclin D3-FITC mAb (MOPC-21; BD Pharmingen) and analyzed by fluorescence microscopy (400 ×).

MAPK and effector phosphorylation analysis by flow cytometry

Cells were fixed in PBS with formalin (2%) for 1 hour and in 70% ethanol overnight (4°C). After washes in PBS–0.5% BSA–Triton 0.25× (PBT), cells were incubated with either anti-MAPK mAbs [Cell Signaling; phospho-p38 Thr180/Tyr182 (clone-12F8), phospho-p42/44 Thr202/Tyr204 (clone-20G11), phospho JNK/SAPK (c-jun N-terminal kinase/ stress-activated protein kinase) T183/Y185 (clone-98-F2)] or rabbit IgG isotype for 45 minutes (4°C). Cells were washed and incubated with a

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secondary anti-rabbit antibody coupled to Alexa Fluor 488 nm (Invitrogen). Phosphorylation levels of Raf, p38 effectors, and Flt3 on gated $CD34^+CD41^+FLT3^+$ cells were determined after labeling with rabbit mAbs included in Raf Family Antibody or Phospho-p38 MAPK Pathway Sampler Kits (nos. 2330 and 9913, respectively) or Tyr591-Flt3 from Cell Signaling, followed by a secondary GAR-Alexa Fluor 633 nm antibody (Invitrogen). About 10^4 events were analyzed on a FACScalibur.

In vitro migration assay

Transwell migration assays were carried out on MK derived from $CD34^+$ cultures (D6) as previously described (27). Cells (10^5) were loaded on the top chamber and rhFL (10–100 ng/mL) was added or not to the bottom chamber, with or without rhSDF-1 (100 ng/mL), and incubated for 48 hours at 37°C in RPMI/0.5%BSA. In some experiments, Flt3 inhibitor IV (42 nmol/L; Calbiochem) or p38 inhibitors (SB203580, 1 μmol/L, SB202190, 40 nmol/L; Sigma) were added to the culture. The percentage of migrating cells was calculated after quantification of live cells in top and bottom chambers.

Western blot

Total cell lysates (10/30 μg), obtained as previously described (28), were subjected to SDS-PAGE, electrophoretically transferred into nitrocellulose membranes, and blotted using primary mAbs similar to those used for cytometric analysis or using actin antibody (Santa Cruz Biotechnology). Membranes were revealed with anti-mouse or anti-rabbit IgG horseradish peroxidase–linked antibodies and a chemiluminescence detection kit (ECL-Plus; GE-Healthcare); signals were quantified by using ImageJ software.

Patient group prediction model

Upstream p38 effectors and *C-MYC* expression modulation in MK derived from $CD34^+$ (D6) in response to 18-hour FL stimulation kinetics was quantified by QRT-PCR. Ratio at each time point of kinetics was normalized as compared with quantification at the starting point of stimulation. Cumulative scores of each kinetic time ratio were included in a multivariate model composed of a principal component analysis associated to a hierarchical clustering in which patient clinicobiological information were reported.

Statistical analysis

Results were expressed as mean ± SD. Statistical differences between patients and controls were validated by unpaired *t* test with a significant $P < 0.05$. Statistical differences between conditions were validated by paired *t* test with significant $P < 0.001$ (***); $P = 0.001$ to 0.01 (**); and $P = 0.01$ to 0.05 (*). Fisher ANOVA test with 2 factors (samples and time of differentiation) was also used; a $P < 0.05$ was significant. NS, not significant.

Results

FL plasma level is increased in PMF patients and is overexpressed in $CD34^+$ hematopoietic and stromal cells

We first showed that FL plasma level was significantly increased in PMF patients as than in HDs (Fig. 1A; 130.1 ± 78.41, $n = 52$ vs. 69.84 ± 30.94, $n = 28$; $P = 0.0001$) with no significant difference between PMF patients according to their Jak2 status (181.4 ± 70.8 pg/mL, $n = 7$ vs. 128.8 ± 81.9 pg/mL, $n = 18$ for $Jak2^{V617F}$ and $Jak2^{WT}$, respectively). The increased FL level appears to be restricted to PMF patients, as it was statistically different from that of polycythemia vera (PV; $n = 17$) and essential thrombocytopenia (ET; $n = 17$) patients.

We further analyzed the nature of FL producing cells and showed that fibroblasts from PMF BM expressed higher FL level than those from HD (1.17 ± 0.4, $n = 8$ vs. 0.56 ± 0.17, $n = 6$, respectively; $P = 0.002$). As $CD34^+$ and MK cells from PMF patients are known to produce cytokines (13, 29), we analyzed whether they expressed FL. Whereas we did not detect FL transcript in PMF or HD MK cells (data not shown), a 2-fold increase in FL mRNA level was observed in PMF $CD34^+$ cells as compared with that in HD cells (Fig. 1A; 0.080 ± 0.218, $n = 22$ vs. −0.294 ± 0.242, $n = 6$; $P = 0.0006$).

Flt3 expression and phosphorylation are increased in PBMNC, MK, and $CD34^+$ cells from PMF patients

Figure 1B shows that Flt3 mRNA level was increased in mononuclear cells from peripheral (PBMNC) from PMF patients than in HD (0.69 ± 0.96, $n = 11$ vs. −0.06 ± 0.47, $n = 16$, respectively; $P < 0.006$). Similar to FL, Flt3 overexpression was restricted to PMF because Flt3 mRNA level was statistically different in PMF as compared with ET and PV. We further analyzed Flt3 expression on PBMNC by flow cytometry and showed that the percentage of cells coexpressing the $CD41^+$ MK antigen and Flt3 was higher in PMF than in HD (Fig. 1B). Western blot analysis confirmed the Flt3 increased expression and evidenced its phosphorylation in PBMNC from PMF patients (Fig. 1B). As Flt3 is reported to be expressed in HP, we analyzed its distribution on freshly purified $CD34^+$ cells. Figure 1C showed that the percentage of $CD34^+Flt3^+$ cells was increased in PMF compared with that in HD cells (17.77 ± 14.98, $n = 11$ vs. 4.72 ± 7.09, $n = 10$, respectively; $P = 0.01$). Flt3 was mainly expressed on PMF MK progenitors, as the proportion of $CD34^+Flt3^+CD41^+$ cells was significantly increased in $Jak2^{WT}$ and $Jak2^{V617F}$ patients than in HD (76.11 ± 14.49, $n = 4$ and 43.37 ± 23.86, $n = 12$ vs. 9.11 ± 10.97, $n = 11$; $P < 0.0001$ and $P = 0.0001$, respectively; Fig. 1D) with a higher proportion in $Jak2^{WT}$ patients ($P = 0.01$). To assess Flt3 activation on this cell subset, we quantified the percentage of $CD34^+CD41^+Flt3^+$ cells expressing phospho-Flt3 and showed that it was increased in PMF patients ($Jak2^{WT}$ and $Jak2^{V617F}$) versus HD (28.21 ± 22.67, $n = 5$ and 18.35 ± 24.81, $n = 4$ vs. 0.22 ± 0.34, $n = 10$; $P = 0.007$ and $P = 0.01$, respectively; Fig. 1D). In accordance with these results, Western blot analysis also evidenced the presence of the Flt3 protein and of its phosphorylated form in freshly isolated $CD34^+$ cells and in MK-derived $CD34^+$ cells from PMF patients (Fig. 1E).

The increased percentage of PMF $CD34^+CD41^+$ MK progenitors expressing Flt3 and its activated form motivated us to analyze its expression during *in vitro* megakaryopoiesis. We quantified by QRT-PCR the Flt3 transcript in MK derived from $CD34^+$ culture (D10) and showed that it was significantly overexpressed in PMF patients as compared with

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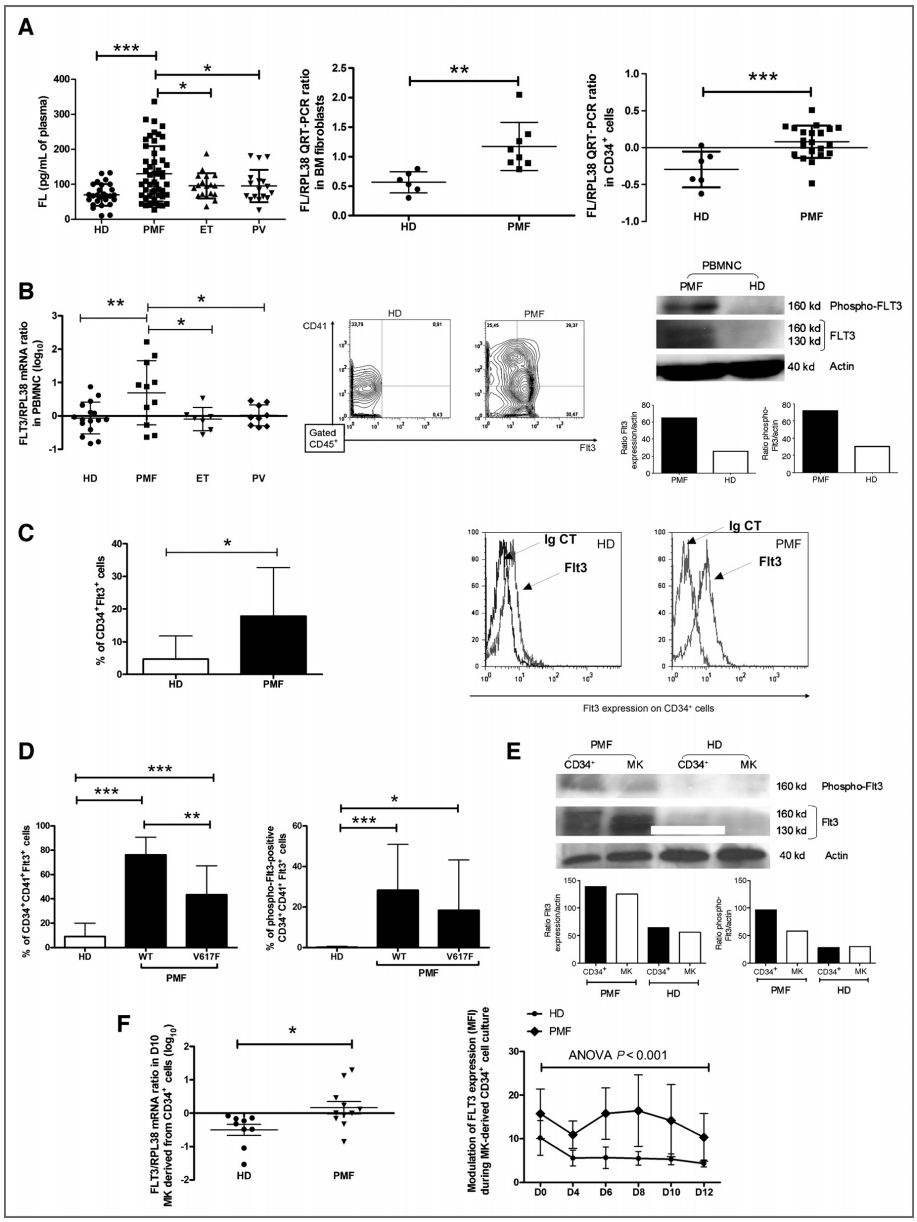

Figure 1. Increased FL level, Flt3 expression, and phosphorylation in PMF patients. A, FL plasma level in HD, PMF, ET, and PV; FL mRNA expression in BM fibroblasts and $CD34^+$ cells from HD and PMF. B, Flt3 mRNA expression in PBMNC from HD, PMF, ET, and PV, Flt3 membrane expression in $CD41^+CD45^+$ PBMNC and Flt3 protein expression and phosphorylation in PBMNC determined by Western blot analysis. C, percentage of circulating $CD34^+Flt3^+$ cells and cytogram of Flt3 expression. D, $CD34^+CD41^+Flt3^+$ cells and of phospho-Flt3–positive $CD34^+CD41^+Flt3^+$ cells in HD and PMF, according to their Jak2 status. E, Flt3 expression and phosphorylation in $CD34^+$ and MK derived from $CD34^+$ cells determined by Western blot analysis. F, Flt3 mRNA expression in MK and modulation of its membrane expression during MK-derived $CD34^+$ culture. *, $P = 0.01$ to 0.05; **, $P = 0.001$ to 0.01; ***, $P < 0.001$.

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HD (0.168 ± 0.615, $n = 11$ vs. −0.498 ± 0.488, $n = 9$; $P = 0.008$; Fig. 1F). We further analyzed the variation of Flt3 membrane expression at different time points during *in vitro* MK differentiation. In contrast to HD, Flt3 expression persisted in patients throughout the culture with a maximal expression between days 6 and 10; the ANOVA/Fisher test showed a significant difference ($P < 0.001$) between HD (BM, $n = 10$; PB, $n = 12$) and PMF patients ($n = 16$; Fig. 1F).

Altogether, these data indicate that Flt3 expression and phosphorylation are increased in PMF $CD34^+$ cells and are maintained during MK differentiation.

MAPK phosphorylation is increased in PMF $CD34^+$ and MK cells and is related to cells expressing Flt3

Binding of FL to Flt3 triggers several downstream signals mainly including PI3K and Ras/MAPK pathways. A profiling gene expression of PMF $CD34^+$ cells had allowed us to generate 2 lists of differentially expressed genes (HD vs. PMF $Jak2^{WT}$ and HD vs. PMF $Jak2^{V617F}$; Gene Expression Omnibus, GEO, no. GSE12234). These lists were used to carry out a functional representation based on a pathway annotation using NIH DAVID software (http://david.abcc.ncifcrf.gov/). This analysis indicated that several genes involved in the MAPK pathway are commonly deregulated in PMF patients (Fig. 2A and Supplementary Table S2). We also evidenced that MAPK pathway gene and especially p38-dependent transcription factors involved in inflammatory process, such as AP-1 and Fos, were altered in PMF MK cells (Supplementary Table S3). We further investigated MAPK pathway deregulation in $CD34^+$ cells and during *in vitro* megakaryopoiesis in PMF patients.

Figure 2B shows that p38, Jnk, and p42/p44 phosphorylation levels were significantly increased in PMF $CD34^+$ cells, independently of their Jak2 status, as compared with that in HD cells: p38 (HD: 7.74 ± 3.67, $n = 21$ vs. PMF $Jak2^{WT}$: 21.08 ± 12.05, $n = 8$; $P < 0.0001$ and vs. PMF $Jak2^{V617F}$: 16.78 ± 8.11, $n = 12$; $P < 0.0001$), p42/p44 (HD: 6.8 ± 2.97, $n = 22$ vs. PMF $Jak2^{WT}$: 16.58 ± 9.84, $n = 8$; $P = 0.0001$ and vs. PMF $Jak2^{V617F}$: 12.11 ± 11.76, $n = 12$; $P = 0.02$), and JNK (HD: 6.17 ± 4.54, $n = 24$ vs. PMF $Jak2^{WT}$: 26.76 ± 19.56, $n = 8$; $P < 0.0001$ and vs. PMF $Jak2^{V617F}$: 13.87 ± 12.15, $n = 12$, $P = 0.005$).

We then analyzed whether MAPK activation was maintained during *in vitro* MK differentiation and showed that, similar to $CD34^+$ cells, increased phosphorylation levels of p38 and Jnk were observed in D10 MK derived from PMF $CD34^+$ culture irrespectively of their Jak2 status (p38: 18.34 ± 11.62, $n = 10$ and 18.27 ± 12.09, $n = 18$, for PMF $Jak2^{WT}$ and $Jak2^{V617F}$, respectively vs. 8.99 ± 5.11, $n = 17$ for HD, $P = 0.0038$ and $P = 0.0030$, respectively; JNK: 21.95 ± 20.98, $n = 10$ and 22.76 ± 25.35, $n = 16$ for PMF $Jak2^{WT}$ and $Jak2^{V617F}$, respectively vs. 6.91 ± 3.96, $n = 17$ for HD, $P = 0.0038$ and $P = 0.0079$, respectively; Fig. 2B). A modest increased p42/p44 phosphorylation was also observed in PMF MK cells.

We further studied the phosphorylation of up- and downstream p38, JNK, and p42/p44 effectors in PMF cells and whether this phosphorylation was associated with Flt3 expression. Figure 2C showed that the percentage of $CD34^+CD41^+Flt3^+$ cells expressing phospho-a/b/c-Rafs or phosphorylated forms of p38 effectors such as MKK3-6, MAPKAPK2, and ATF2 as well as of MSK1 and HSP27 were significantly increased in PMF patients whatever their Jak2 status ($Jak2^{V617F}$, $n = 4$ and $Jak2^{WT}$, $n = 5$) as compared with HD (PB, $n = 6$ and BM, $n = 3$).

Therefore, our data showed that MAPK phosphorylation is increased in PMF $CD34^+$ and MK cells and is related to Flt3 expressing cells.

Flt3-dependent p38–MAPK pathway deregulation in PMF $CD34^+$ and MK cells

Among MAPKs, p38 is strongly responsive to stress and inflammatory mediators such as TNF-α, IL-1β, and IL-8 known to be highly expressed in PMF (30); so, we further focused our study on p38–MAPK and on its main up and downstream effectors. We confirmed that p38 was phosphorylated in $CD34^+$ and MK cells from patients by Western blot analyses (Fig. 3A), and that p38 phosphorylation was associated with MK expressing Flt3 (Fig. 3B). p38 phophorylation was associated with activation of its upstream MKK3-6 effector and with upregulation of downstream AP-1 target gene expression in both $CD34^+$ and $CD41^+$MK derived from $CD34^+$ cells (Fig. 3C and D) reinforcing the notion that p38 axis is activated in PMF cells.

To analyze the role of Flt3 in p38–MAPK pathway activation in PMF $CD41^+$MK derived from $CD34^+$ culture, we quantified the phosphorylation of p38–MAPK effectors after addition of a neutralizing Flt3 antibody (10 μg/mL) to PMF cells from 6 patients. We showed that such treatment reduced phosphorylation of up- and downstream targets of p38 (Fig. 2E). Finally, to ascertain the implication of Flt3 in p38–MAPK activation, we showed that silencing 50% of Flt3 protein expression (Fig. 3F) and 80% of its transcript (data not shown) in MK derived from $CD34^+$ resulted in a decreased percentage of phospho-$p38^+$ cells (Fig. 3G).

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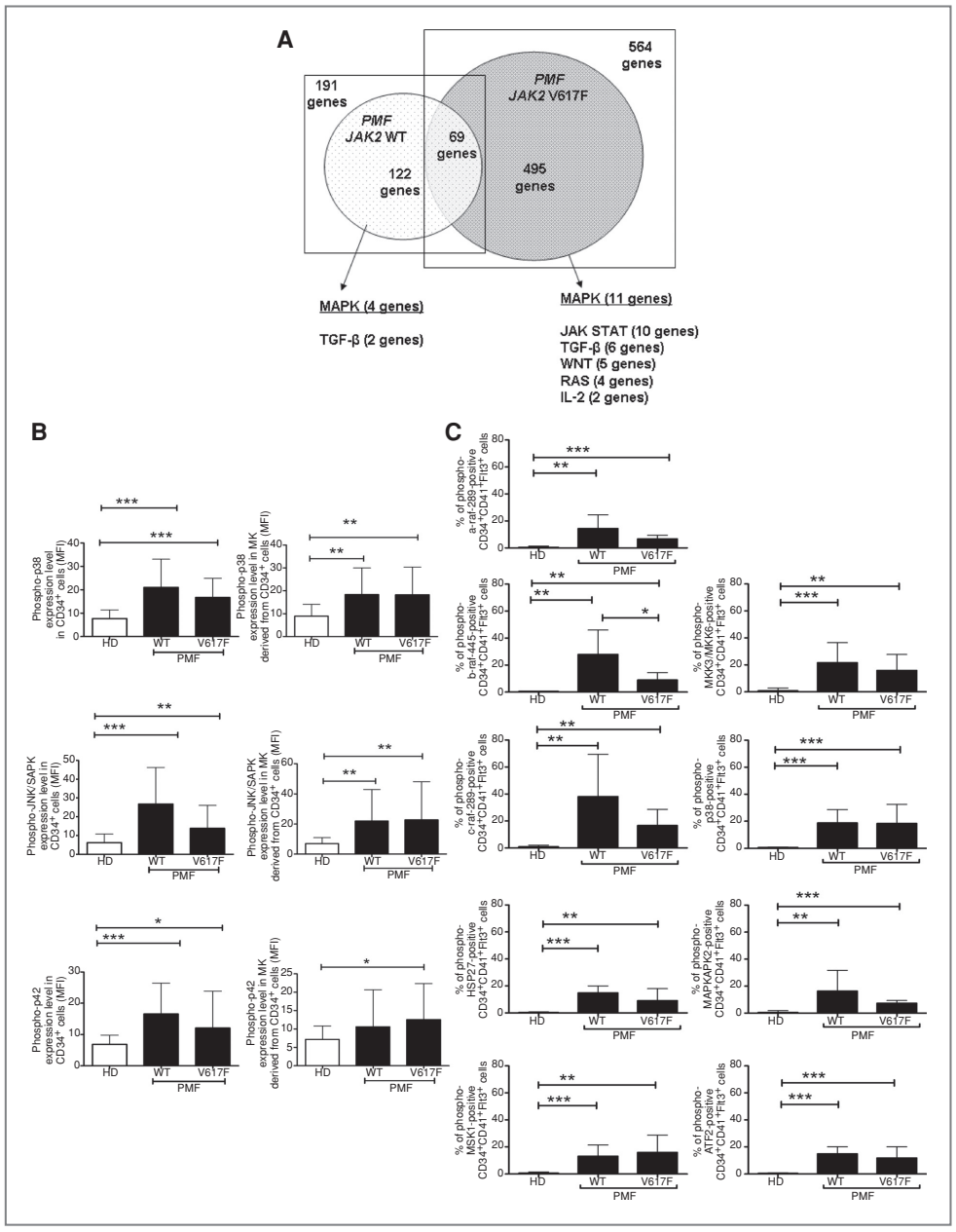

Figure 2. MAPK effector expression and phosphorylation in CD34$^+$ cells and MK cells from PMF patients. A, Venn diagram showing MAPK deregulation in PMF CD34$^+$ cell transcriptome. Phosphorylation level (MFI) of p38, JNK/SAPK, and p42/p44 in CD34$^+$ and MK derived from CD34$^+$ cells (B) of Raf (a-raf-289, b-raf-445, c-raf-289) and p38 pathway effectors (MKK3-6, MSK1, MAPKAPK2, ATF2, and HSP27) in CD34$^+$Flt3$^+$CD41$^+$ MK progenitors (C) from HD and PMF patients according to their Jak2 status. *, $P = 0.01$ to 0.05; **, $P = 0.001$ to 0.01; ***, $P < 0.001$.

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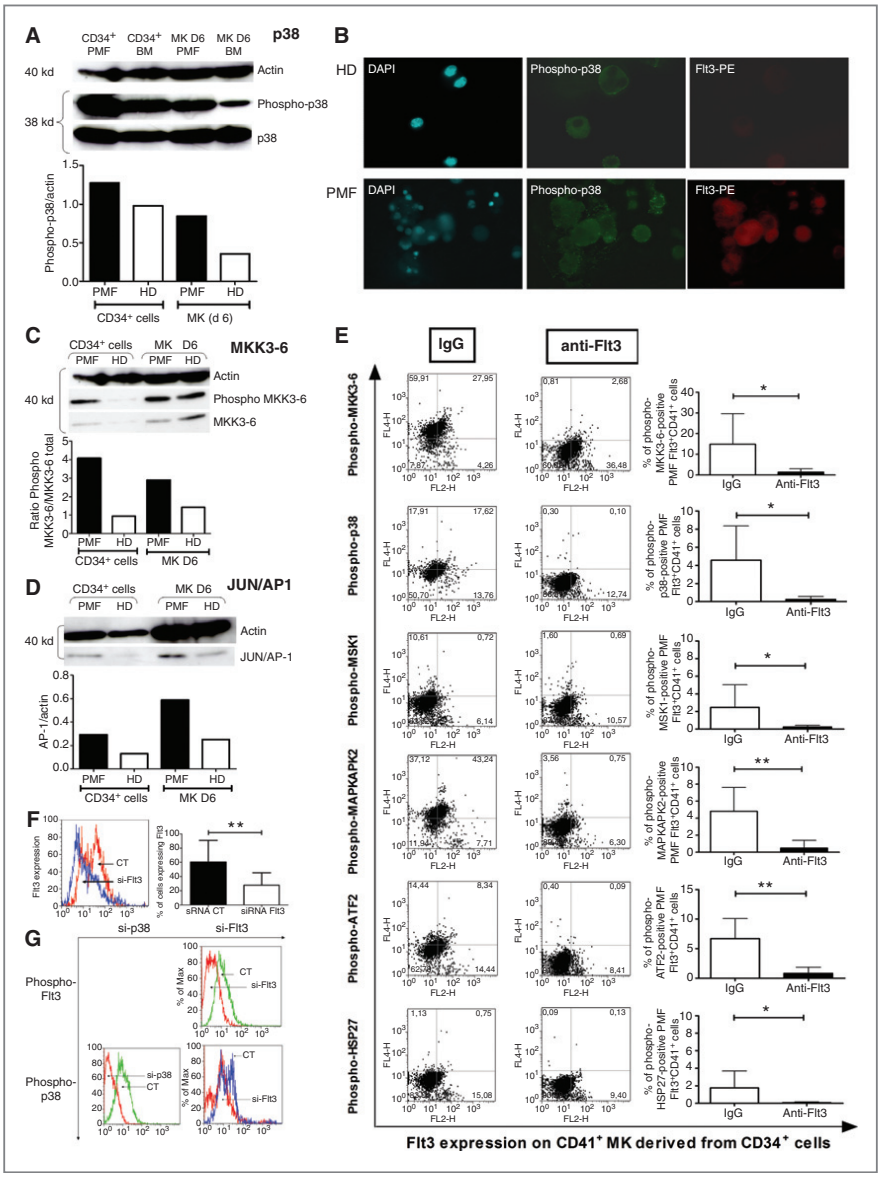

Figure 3. Flt3-dependent p38–MAPK effector phosphorylation in PMF megakaryopoiesis. A, phospho-p38 determined by Western blot analysis. B, p38 phosphorylation and Flt3 expression in MK derived from HD or PMF CD34$^+$ cells (day 10) by immunocytology (650×). C, phospho-MKK3-6 determined by Western blot analysis. D, expression level of JUN/AP-1 determined by Western blot analysis. E, effect of anti-Flt3 mAb on p38 effector phosphorylation (MKK3-6, MSK1, MAPKAPK2, ATF2, and HSP27) in Flt3$^+$CD41$^+$MK derived from PMF CD34$^+$ cells (day 10). F, Flt3 expression in MK derived from PMF CD34$^+$ cells after Flt3 silencing as determined by flow cytometric analysis. G, effect of p38 and Flt3 RNA silencing on their respective phosphorylations in MK derived from PMF CD34$^+$ cells (day 8). DAPI, 4′,6-diamidino-2-phenylindole. *, P = 0.01 to 0.05; **, P = 0.001 to 0.01.

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confirm the triggering effect of FL/Flt3 on p38 pathway, we further analyzed by flow cytometry the activation of ATF2, a key cross-talk molecule for p38 transcriptional activity, in response to FL stimulation. Figure 4C shows that ATF2 phophorylation level is increased in MK derived from PMF $CD34^+$ in response to FL in a time-dependent manner. In contrast, there is no ATF2 phosphorylation in response to FL stimulation in HDs.

We further analyzed whether the expression of MAPK-associated downstream transcription factors was modulated in response to FL. Figure 4D shows that p38-associated p53, NFATc4/NFAT3, AP-1, and ATF2 transcripts are upregulated after FL stimulation in PMF but not in HD samples. In contrast, MSK2, PARK, MK3, MNK1, MNK2, and MSK1 transcripts that are Erk (extracellular signal–regulated kinase)-dependent transcriptional factors were not affected by FL stimulation either in HD or in PMF (Supplementary Table S4). Interestingly, the transcript of IL-8, known to be involved in PMF dysmegakaryopoiesis (30) and to be stabilized by p38 (31), showed a rapid increase in response to FL in PMF cells (Fig. 4D). This IL-8 overexpression was reduced after Flt3 or p38 silencing (data not shown) strengthening the role of the Flt3/p38 signaling in IL-8 regulation.

Altogether our data confirm that FL *in vitro* activates p38 cascade and increases its target gene expression through Flt3 in PMF MK–derived $CD34^+$ cells.

Modulation of upstream MAPK linker expression in response to FL allows definition of PMF patient group prediction

We further searched whether p38–MAPK upstream linker expression was modified in response to FL in PMF MK–derived $CD34^+$ cells and whether modulation of their expression could predict exaggerated FL/MAPK axis response. We have principally selected upstream MAPK linkers proximal to Flt3, minimizing the influence of other pathways such as PI3K and JAK/STAT (Fig. 5A) and quantified their mRNA expression at different time points of FL kinetics, by QRT-PCR. Data (Supplementary Table S5) were used to draw a gene neuronal network that shows a good correlation between genes selected and validates their choice for the predictive model (Fig. 5B). A three-dimensional (3D) projection plot of principal component multivariate analysis (PCA) showed that HD samples (BM, PB) are closely related in the centre of the plot. The PMF4 patient, who is *de novo* diagnosed, is close to the HD aggregate, in contrast to the 3 other patients (PMF1, PMF2, and PMF3) who were earlier diagnosed (Fig. 5C). These PCA dispersion data incited us to build a hierarchical classification plot correlating this clustering to clinicobiological data (Fig. 5D). This classification allowed to distinguish HD and PMF groups and to identify PMF subgroups correlated with clinical data such as Jak2 mutation status, myeloproliferation, and Dupriez score.

This predictive model confirms the implication of Flt3/MAPK axis activation in PMF megakaryopoiesis and establishes a link with patient clinicobiological parameters.

Flt3/MAPK axis is involved in PMF dysmegakaryopoiesis

Our results showing that Flt3/MAPK axis was altered in PMF megakaryopoiesis raise the question of whether it is involved in this pathologic process (30). Therefore, we assessed the effect of p38, p42/p44, Jnk, and Flt3 inhibitors (refs. 32, 33; Supplementary Table S6) or of anti-Flt3 mAb (10 μg/mL) on PMF MK–derived $CD34^+$ cultures. Inhibition of either MAPK or Flt3 significantly reduced the proliferation of MK derived from PMF $CD34^+$ cells (Fig. 6A), without affecting their viability (Fig. 6B). Figure 6C showed that such treatment increased the percentage of polyploid MK derived from PMF $CD34^+$ cells, especially of cells with a ploidy more than 64N (Supplementary Table S6) and induced a nuclear localization of cyclin D3, known to promote endomitosis. It also stimulated MK differentiation as shown by an increased expression of the CD41 MK differentiation marker (Fig. 6D). A similar effect on CD41 expression was observed after p38 or Flt3 silencing, confirming their participation in the differentiation process (Fig. 6D and Supplementary Fig. S1). Furthermore, Flt3 inhibitors restored the formation of proplatelets (Fig. 6D). Participation of the Flt3/p38-mediated pathway in the control of PMF megakaryopoieis was further confirmed by results showing an upregulation of GATA-1, FOG-1, FLI-1, NFE2 MK transcriptional factors, of Aurora B endomitotic regulator and of TGFB1, PF4, and CD9 MK marker gene expression after Flt3 or p38 silencing (Fig. 6E).

FL has been reported to be a mobilization factor (27). Therefore, we analyzed whether Flt3/p38 axis participates in PMF MK migration in response to FL. We first determined FL doses that induced a maximal MK migration (data not shown). In contrast to HD, in PMF patients, addition of 10 ng/mL FL induced a significant MK precursor migration as compared with untreated cells (46.85% ± 10.09% vs. 25.88% ± 5.29%; $n = 3$, $P = 0.03$; Fig. 6F). FL-mediated migration was inhibited by addition of Flt3 inhibitor (Fig. 6F), confirming the role of Flt3 in the PMF FL–induced migration. Addition of p38 inhibitors also totally inhibited the FL-mediated migration (Fig. 6F) showing that it was dependent on p38 activation. As expected, addition of SDF-1 alone used as control, stimulated the migration of MK precursors from either PMF patients or HD (Fig. 6F). However, when added to FL, SDF-1 did not increase the PMF FL–induced migration (Fig. 6F), suggesting that both processes are differentially regulated in patients.

Altogether, our data support the notion for a role for the Flt3/p38-MAPK axis in PMF dysmegakaryopoiesis.

Discussion

PMF is characterized by a clonal amplification of HSC, an increased circulating $CD34^+$ cell number, and a prominent MK proliferation with altered maturation and dysplastic features in the bone marrow. This myeloproliferation is associated with marked changes in the BM stroma characterized by myelofibrosis, osteosclerosis, and neoangiogenesis consequent to an overproduction of fibrogenic and inflammatory cytokines by hematopoietic cells and especially by MKs (34). Reciprocally, studies from our group have shown the role of stromal cells from PMF patients in the myeloproliferation and especially in

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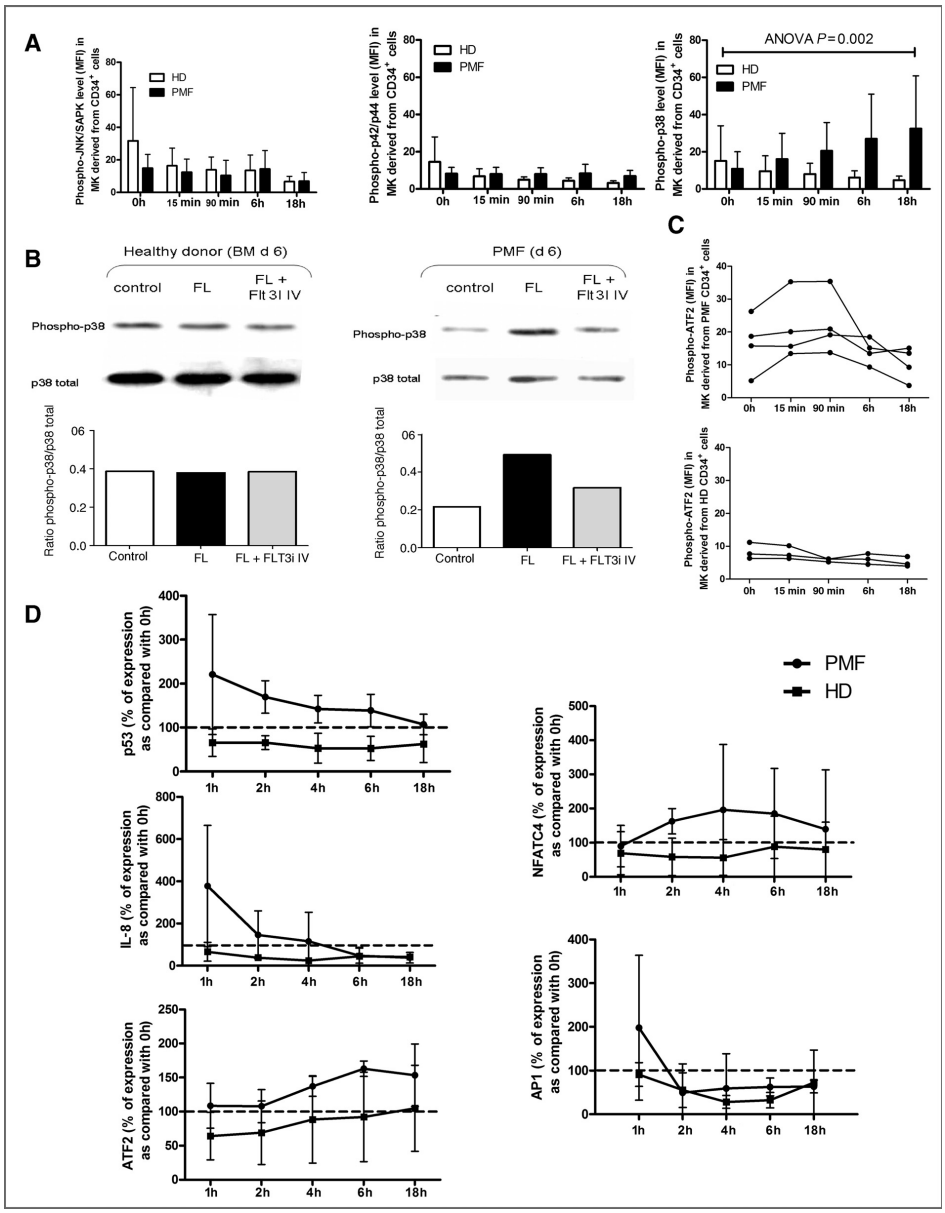

Figure 4. Modulation of p38–MAPK effector phosphorylation and MAPK downstream regulator expression in PMF MK–derived CD34$^+$ cells in response to FL stimulation. These stimulations were carried out at 50 ng/mL. A, effect of 18-hour FL kinetic on the phosphorylation level (MFI) of p-38, JNK/SAPK, and p42/p44 in PMF MK derived from CD34$^+$ cells (day 6). B, phospho-p38 in HD and PMF MK derived from CD34$^+$ cells (day 6) in response to 18-hour FL stimulation and Flt3 inhibitor treatment determined by Western blot analysis. C, modulation of ATF2 phosphorylation in response to a time-dependent FL stimulation determined by flow cytometric analysis. D, transcriptional regulation of MAPK effector (ATF2) and transcription factors as well as IL-8 after 18-hour FL kinetic in HD and PMF MK derived from CD34$^+$ cells (day 6).

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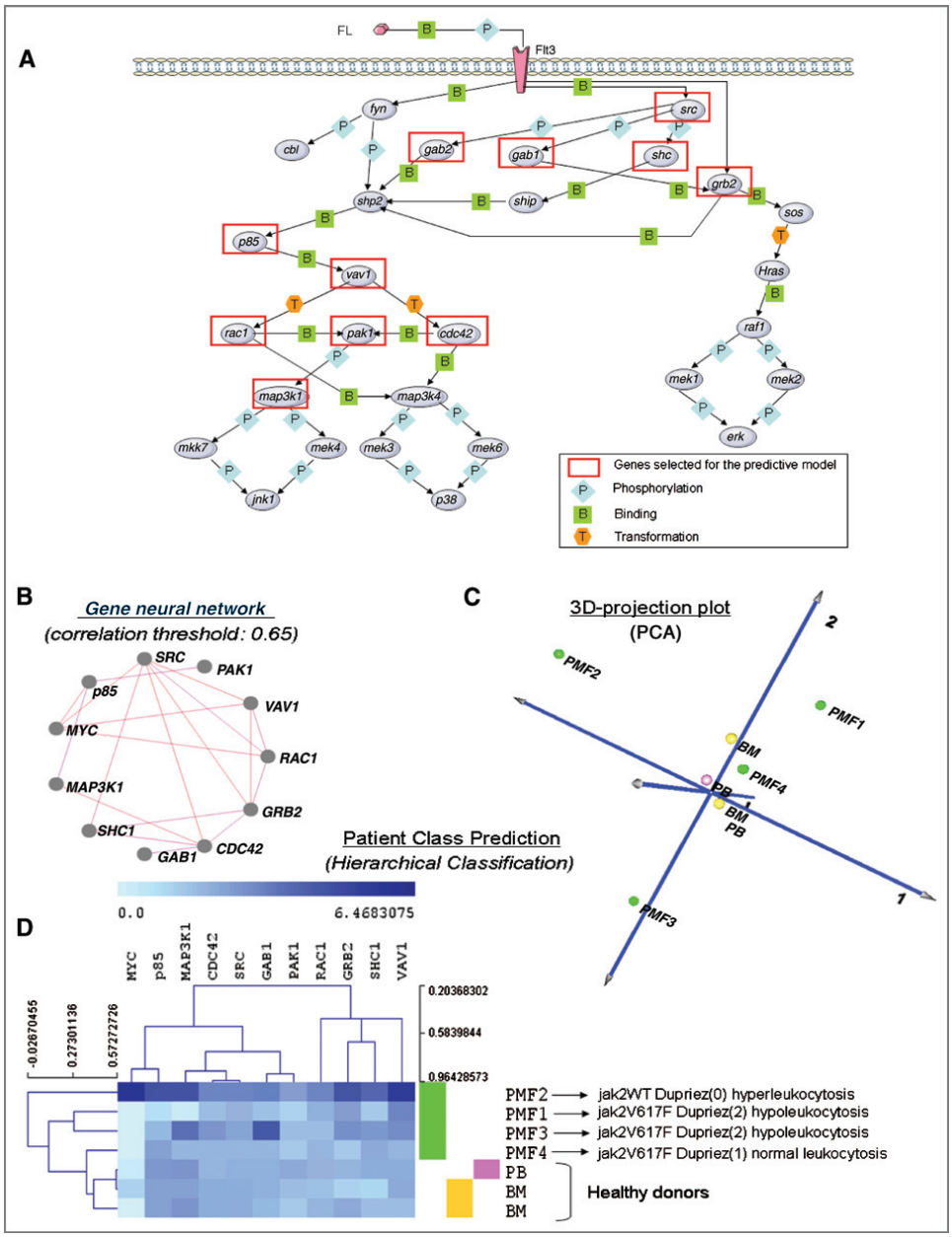

Figure 5. PMF patient group prediction model according to modulation of upstream MAPK linker and effector expression in response to FL stimulation. A, scheme of genes involved in Flt3/MAPK axis drawn from www.genego.com. Red frame genes were selected for conducting a predictive model of PMF patient groups according to FL-dependent MAPK response (B–D). Predictive model: (B) neural network with selected genes, (C) principal component analysis, (D) hierarchical clustering with correlation of clinicobiological data.

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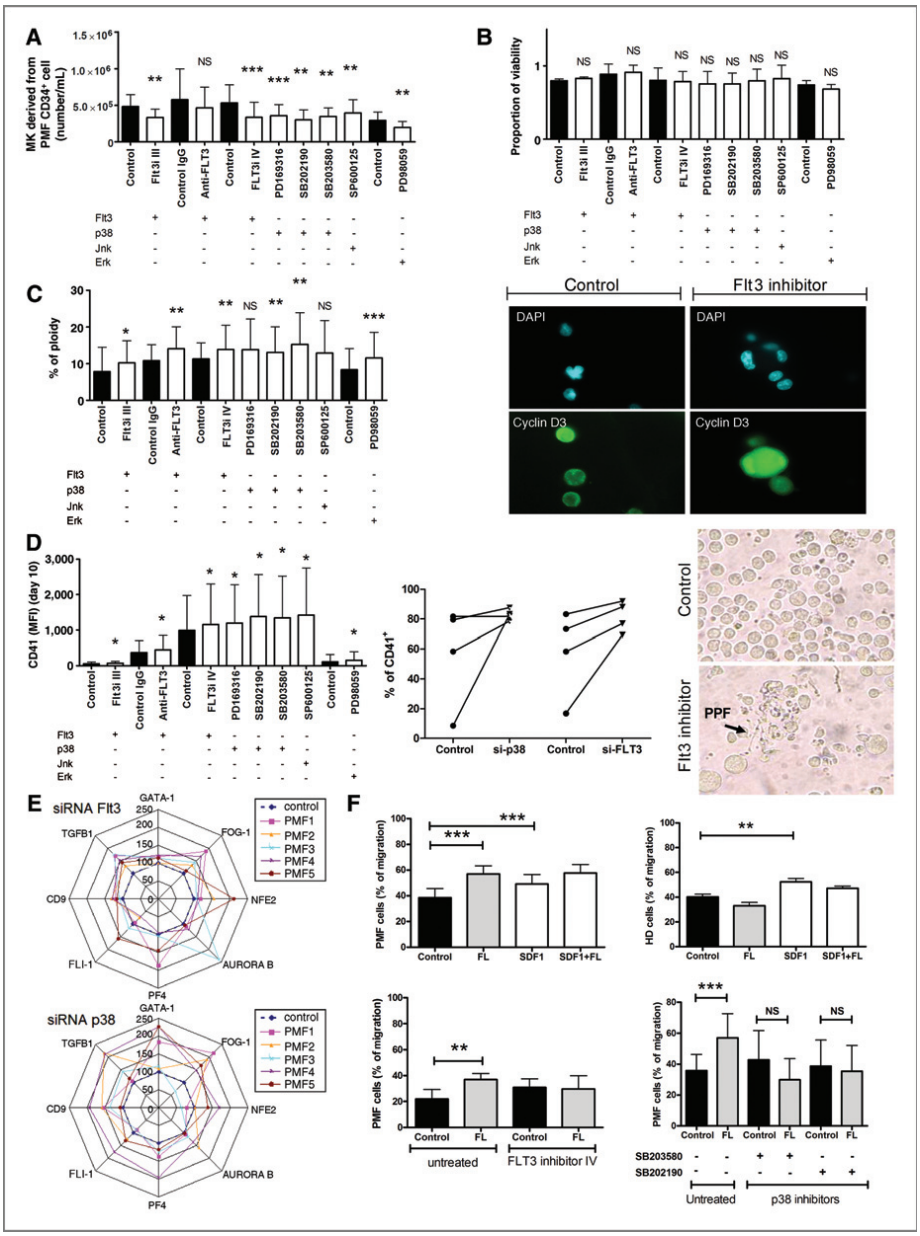

Figure 6. Flt3/MAPK axis is involved in PMF dysmegakaryopoiesis. Effect of Flt3 or MAPK inhibitors on proliferation (A) and viability (B) of PMF MK derived from $CD34^+$ culture (D10), polyploidy cell percentage, cyclin D3 expression and cytologic maturation (400×; C). D, effect of inhibitors and siRNA on CD41 expression and proplatelet formation. E, effect of Flt3 and p38 silencing on gene expression involved in MK regulation quantified by QRT-PCR. F, percentage of migration of MK derived from $CD34^+$ cells purified from HD or PMF patients in response to FL (10 ng/mL) in the presence or absence of SDF-1α (100 ng/mL) and effect of Flt3 and p38 inhibitors on PMF MK derived from $CD34^+$ cell migration in response to FL (10 ng/mL). *, $P = 0.01$ to 0.05; **, $P = 0.001$ to 0.01; ***, $P < 0.001$; ns, not signficant.

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the dysmegakaryopoiesis that characterizes the disease (35). Among cytokines secreted by stromal cells, FL (36) has been reported to play a role in HSC proliferation and survival (37). Its receptor, Flt3, is frequently altered in leukemia through gene rearrangements such as ITD that induces a myeloproliferative syndrome in knockin mice model (23). Evidence for such a myeloproliferative phenotype incited us to study the potential involvement of the FL/Flt3 couple in PMF pathogenesis.

Our present data show that the FL plasma level is specifically increased in PMF patients, in whom it is produced by stromal and hematopoietic cells. Furthermore, in contrast to HD in whom Flt3 expression is restricted to HSC/HP and granulomacrophagic progenitors (22), in PMF patients, Flt3 expression and phosphorylation are associated with MK differentiation, as shown by an increased percentage of circulating $CD34^+Flt3^+$ cells expressing the CD41 MK antigen as well as by the increased Flt3 expression on $CD41^+$MK cells. Flt3 overexpression is maintained during $CD41^+$MK derived from PMF $CD34^+$ cell culture, consistent with sustained Flt3 expression along the MK lineage in patients.

Whereas we have shown that Flt3 overexpression is $Jak2^{V617F}$ independent, its mechanism is still unknown. Actually, we did not find any mutations of the *Flt3* gene sequence that could lead to the receptor activation, being compatible with a recent study in PMF patients (38). Among other hypotheses, mutations in the *c-Cbl* gene, an adapter protein that regulates the ubiquitination of receptor protein tyrosine kinases, recently reported in PMF (39), could participate in maintaining Flt3 membrane expression. Flt3 deregulation could be also secondary to other signals including epigenetic modifications, as already shown for CXCR4 in PMF $CD34^+$ cells (40).

Evidence for specific alterations of the FL/Flt3 couple in PMF patients encouraged us to study its potential role in the dysmegakaryopoiesis that characterizes the disease. Among the different mechanisms proposed to be involved, NF-κB (41) activation and IL-8 (30) overexpression are reported to participate in this dystrophic process. $Jak2^{V617F}$ and $MPL^{W515L/K}$ mutations that induce enhanced STAT signaling (42), are suggested to indirectly activate MAPK pathways, known to be important in MK differentiation and especially in endomitosis (24). Our present data, showing in PMF MK cells (i) an increased expression of Flt3 and its phosphorylation, (ii) a modulation of gene profiling involved in MAPK signaling, (iii) an increased phosphorylation of p38, p42/44, and JNK MAPKs, (iv) an increased phosphorylation of p38 pathway effectors, and (v) an activation of Flt3/p38-MAPK axis and an increase of p38 target genes in response to FL, establish a link between Flt3 and MAPK activation, and especially p38, in PMF MKs.

Participation of Flt3 signaling in the PMF dysmegakaryopoiesis through MAPK pathways is suggested by our results showing that treatment with MAPK inhibitors or p38 RNA silencing reverses MAPK phosphorylation and restores MK differentiation. Our hypothesis that a sustained MAPK pathway activation participates in MK maturation block observed in PMF is also supported by our data showing that FL stimulation of PMF MK precursors provokes an increase of p38, recently reported to play a role in PMA (phorbol-12-myristate-13-acetate)-induced MK differentiation of K562 cells (43). The presence of phospho-p38 in the cytoplasm of polyploid MKs (Fig. 3B) strongly suggests its activation in those cells (44). It is also consistent with the stabilization of transcripts with 3′UTR (untranslated regions) AU-rich element-motif (ARE-motif; ref. 31) like IL-8, shown to be upregulated in response to FL (Fig. 4D) and to participate in PMF dysmegakaryopoiesis (30). The p38 phosphorylation recently reported in BM sections from MPN patients (45) confirms our data obtained in culture and supports our hypothesis on p38 activation in PMF.

As expected, FL-induced MAPK phosphorylation is not observed in HD MK precursors which no longer express membrane Flt3. In contrast, in PMF patients, we suggest that an elevated FL level maintains MAPK activation in MK precursors which abnormally expressed Flt3. Our data, showing that Flt3 inhibition by either antibodies or gene silencing improves the megakaryopoiesis in PMF $CD34^+$ cultures and reduces the p38–MAPK effector phosphorylation, reinforce the role of Flt3/p38 axis in PMF dysmegakaryopoiesis. As IL-8 transcripts are suggested to be upregulated by FL stimulation via p38 activation, it can be speculated that phospho-p38 may participate in inflammation observed in PMF by stabilizing transcripts for cytokines/chemokines participating in this process (31). AP-1 and NFATc4 that are upregulated during megakaryopoiesis (46), are overexpressed in response to FL in MK-derived PMF $CD34^+$ cells. Being partners in transcription, they likely cooperate to induce transcription of genes involved in such inflammatory process (47).

In PMF, $CD34^+$ cells egress from the bone marrow to circulate and invade spleen and liver where an extramedullary hematopoiesis is developing. Several mechanisms, including disturbance of SDF-1α-CXCR4 axis (48) and increased extracellular matrix proteolytic activity reducing HP adhesion to BM stroma (49), can explain this migration. Besides its role in primitive hematopoiesis, the FL/Flt3 couple has been reported to regulate cell migration (27). Our data showing that FL stimulates the *in vitro* migration of PMF MK precursors support the hypothesis that the FL/Flt3 couple also participates in the abnormal migration of MKs. In our experimental conditions, the FL-dependent migration PMF MKs is not modified by addition of SDF-1α, suggesting that both processes are differentially regulated in those cells. This FL-dependent migration process is p38 dependent, leading to the assumption that targeting p38 could reduce Flt3-expressing cell mobilization in patients.

Up to now, the general concept in PMF is that stromal changes are secondary to the proliferation of hematopoietic cells and especially of MKs. Recently, we have proposed that an abnormal dialogue between hematopoietic and stromal cells, resulting from microenvironmental niche alterations, participates in the hematopoietic deregulation that characterizes PMF (13). Our present demonstration that FL, overproduced by stromal cells, participates in the altered megakaryopoiesis, supports this hypothesis (35).

In conclusion, our results suggest that activation of the Flt3/MAPK pathway and especially of p38–MAPK participate in PMF dysmegakaryopoiesis, including alterations of proliferation/differentiation and migration processes within an inflammatory context (Fig. 7).

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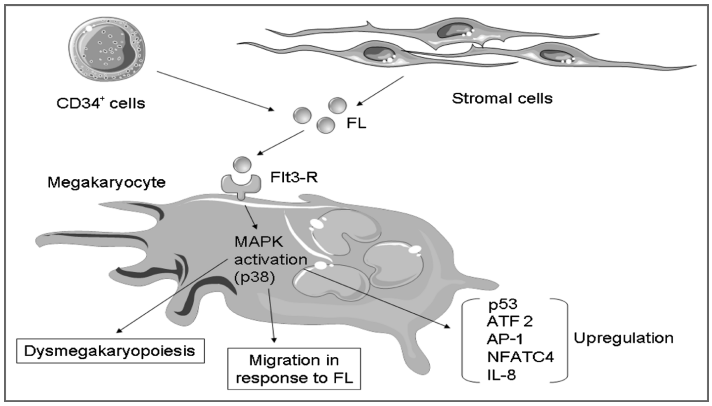

Figure 7. Potential role of Flt3/MAPK axis deregulation in PMF pathogenesis. Pathophysiologic model integrating the "FL/Flt3" couple in PMF MK deregulation through persistent p38–MAPK activation.

The recent therapeutic strategies for PMF mainly target the Jak2 kinase and some of the Jak2 inhibitors also inhibit the Flt3 kinase (50). The clinical efficacy of these inhibitors has been ascribed to a general downregulation of inflammatory cytokine production and signaling (29). Our data suggest that this anti-inflammatory effect could be mediated, at least partially, by the FL/Flt3/p38 axis. In this context, our patient group predictive model, based on FL/MAPK response in MKs and including clinicobiological data, could be powerful in the therapeutic decision to use inhibitors also targeting the Flt3 kinase.

Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interest.

Acknowledgments

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Also, the name of the 20th author appeared incorrectly on the online journal. The correct surname of this author is Le Bousse-Kerdilès. This error has been corrected.

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Cancer Research

Correction: FLT3-Mediated p38–MAPK Activation Participates in the Control of Megakaryopoiesis in Primary Myelofibrosis

In this article (Cancer Res 2011;71:2901–15), which was published in the April 15, 2011 issue of *Cancer Research* (1), Figure 1E contains an error. A corrected version of Figure 1 appears below.

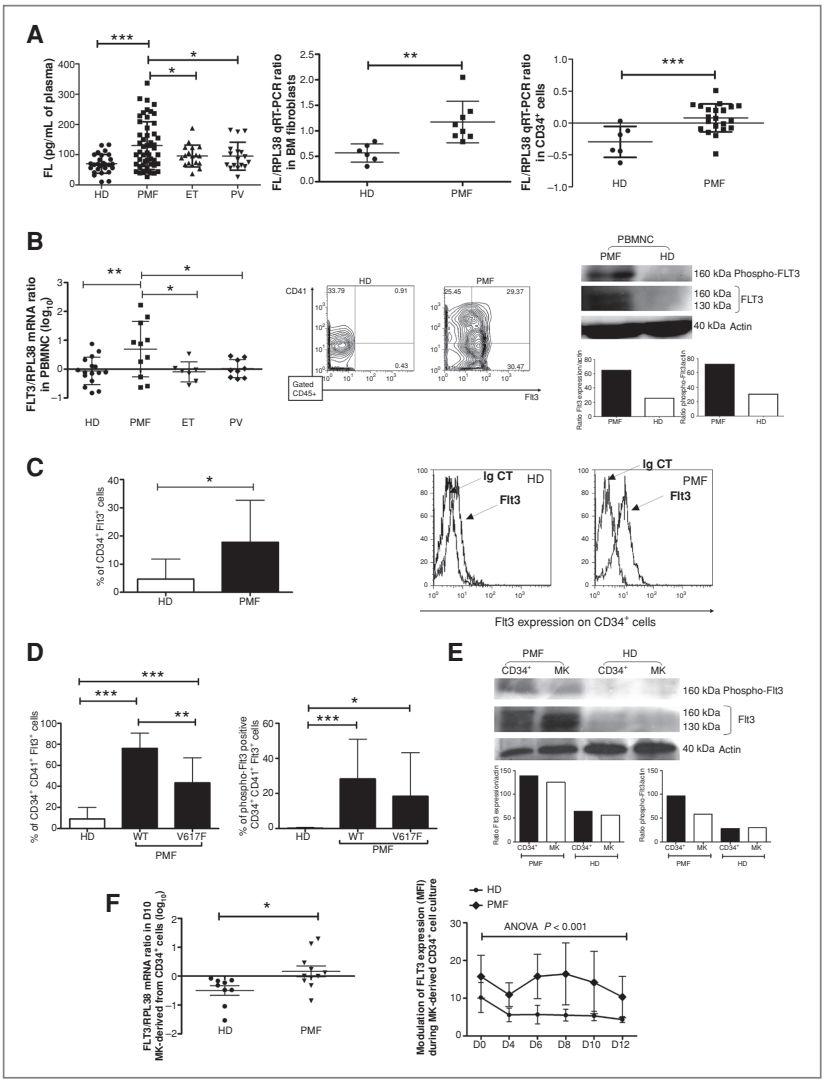
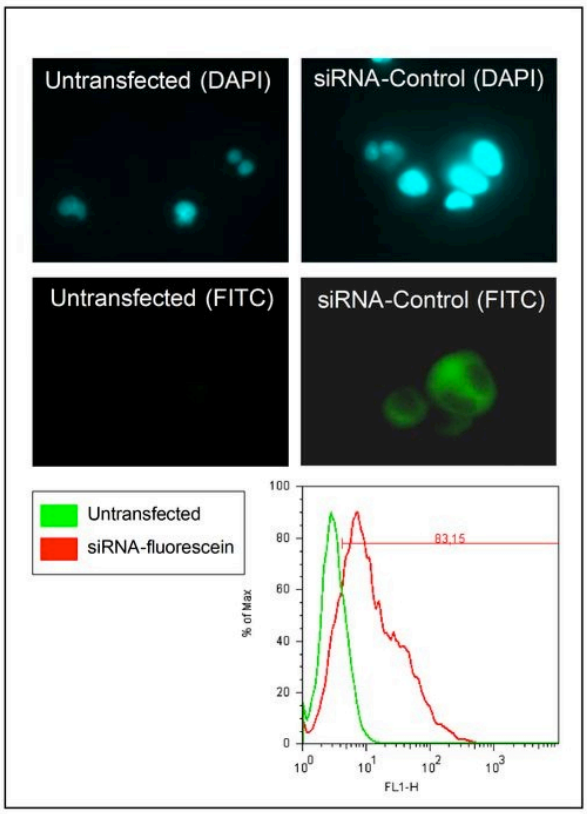


Figure 1.



Supplementary figure 1: Validation by microscopy and flow cytometry of siRNA transfection in MK-derived from CD34+ cells

Supplementary material

Microarray analysis: Total RNA from $CD34^+$ cells or megakaryocytes was extracted by using TRIZOL (Invitrogen, Cergy Pontoise-France). Quality control of the total RNA was performed by using bioanalyser 2100 (Agilent Technologies); only samples with an intact ribosomal profile were included in the study. Probes were amplified by the linear amplification kit (Agilent technology) and were labelled by either cyanin 5 (Perkin Elmer) for tested samples or cyanin 3 (Perkin-Elmer) for reference probes corresponding to the pool of all samples. Spot intensity extraction after microarray scan and data normalization by the LOWESS method (locally weight scatterplot smoothing) were carried out on the Gustave Roussy Institute platform by Features Extraction software (Agilent technology). Microarray data were submitted to the GEO public database (n° GSE12234).

Normalized intensities were transformed into log2 ratio to be analyzed by TM4 MultiExperimental Viewer software (TMEV version 2.0). The transcriptome of BM $CD34^+$ cell was compared to that of PB $CD34^+$ cells from unmobilized healthy donors by Significance Analysis of Microarray (SAM) algorithm with a fold change >2 and False Discovery Rate (FDR) <1 (Figure 1A). This allowed us to build a Venn diagram and to determine a PMF expression profile common to $JAK2^+$ and $JAK2^-$ patients (69 genes). The microarray data of this PMF gene list were loaded in TMEV2.0 software to verify the prediction class of microarray experimental groups by using an unsupervised ascending hierarchical clustering algorithm. The functional analysis of signaling pathways on transcriptome gene lists was carried out with DAVID NIH 2007 software.

Legends of Supplementary Figure 1: Validation by microscopy and flowcytometry of siRNA transfection in MK-derived from $CD34^+$ cells

Picture (X 650) and cytogram show fluorescence of cells transfected or not with FITC siRNA control.

oligo	position	sequence	length (bp)	TM (°C)	GC%
NFATC4					
	foward	gCACGgTATCACAggCAAgA	20	65.0	55
	reverse	gCAgCAgAgTCATCTCCAACA	21	64.8	52
MEFC2					
	foward	CTggCAACAgCAACACCTACA	21	65.55	52
	reverse	AgAAggCAgggAgAgAgATTTgA	21	63.04	47
CHOP					
	foward	CCACTCTTgACCCTgCTTCTC	21	64.8	57
	reverse	gTTTCCTggTTCTCCCTTggT	21	64.4	52
MK2					
	foward	ACATCTgCCTCCTCTCCTCCT	21	66.5	57
	reverse	gCACCTTgCTCACTTCTCTCC	21	65.37	57
PARK					
	foward	CCAgCCAAgTAACAAAgCAgA	21	63.23	47
	reverse	ggCAAATCCAAAgTCACACAA	21	61.98	42
MK3					
	foward	gAgTgAgCgAgTAAgggAggA	21	65.19	57
	reverse	CCAAggAggAgTgAgAATgAgA	22	63.27	50
MNK1					
	foward	CAgTgTCAgggAAACAAgAACA	22	63.02	45
	reverse	ggATgTgggCTAAgATggAACC	21	62.89	52
MNK2					
	foward	gTTTTCAgggAggTggAgATg	21	62.76	52
	reverse	ggATgTggCTCAggATggAg	20	64.57	60
MSK1					
	foward	gCAgAAgAgTTCACAgAAATggA	23	63.07	43
	reverse	ATggAAggAgCAACAAAggAA	21	62.31	42
MSK2					
	foward	gACgAggACgACgATgAgAg	20	64.17	60
	reverse	CCAggAACACCTTgCCgTAg	20	65.28	60
ETS1					
	foward	TCATCTTTCTgCTggTTgTgAg	22	62.88	45
	reverse	CACCCCTCCTCCTTATCCTTT	21	63.12	52
ATF2					
	foward	ATggTAgCggATTggTTAggA	21	63.16	47
	reverse	ggTCTgTggAgTTgTgTgAg	21	64.99	57
AP1					
	foward	CAAgAACTCggACCTCCTCAC	21	64.49	57
	reverse	TCCTgCTCATCTgTCACgTTC	21	64.49	52
P53					
	foward	gTgAgggATgTTTgggAgATg	21	62.83	52
	reverse	CCTggTTAgTACggTgAAgTgg	22	64.63	54

oligo	position	sequence	length (bp)	TM (°C)	GC%
FLT3					
	foward	CgATgTATCAgAATgTggATgg	22	60.96	45
	reverse	TgAgCCTgCggAgAgAgTAg	20	65.41	60
FLT3LG					
	foward	gCTTgTggAgCACTgACCTg	20	65.83	60
	reverse	TTCTgggggAggAATgAgAg	20	62.89	55
CBL					
	foward	CTCgTgTTgATTTgCTgTggT	21	63.79	47
	reverse	TTgATggCggTCTACTgTTTg	21	63.00	47
CDC42					
	foward	CCgCTgAgTTATCCACAAAACA	21	63.00	47
	reverse	AgTCCCAACAAgCAAgAAAgg	21	63.18	47
FYN					
	foward	gAgACAggTTACATTCCCAgCA	22	64.37	50
	reverse	CACTCTCgCggATAAgAAAgg	21	62.89	52
GAB1					
	foward	TCAAgCCCAAAggAgACAAAC	21	63.18	47
	reverse	TggTCAACAACAACATAATCCACTC	25	63.65	40
GAB2					
	foward	AgCGTTCTTCACCCACTTCC	20	64.32	55
	reverse	TAATCCAACCTCCCATTCCAC	21	61.89	47
GRB2					
	foward	AAgCCTgTCCTCACCgTCTC	20	66.21	60
	reverse	ATgACTTCCTCCTCCgCTCTC	21	65.55	57
MAP3K1					
	foward	TCCATCTCgTAACACCATCCA	21	63.07	47
	reverse	gCCCAACAAgCAAAATAggACA	21	63.58	47
MAP3K4					
	foward	gCTgCTCCTgTTTgTCTgATg	21	63.92	52
	reverse	CTACgCTgATgTCACggTTCT	21	64.53	52
p85					
	foward	ACTCACCTTCTgCTCCgTTg	20	64.59	55
	reverse	CTggTCCTgCTggTATTTgg	20	62.89	55
PAK1					
	foward	ggAggCAACAAAgAACAATCAC	22	62.59	45
	reverse	ATCAggAAATggAgAgCAA	21	61.82	42
RAC1					
	foward	CCCgTgAAgAAgAggAAgAgA	21	63.32	52
	reverse	CAAAgCgTACAAAggTTCCAAg	22	62.57	45
SHC1					
	foward	AgTCTggTgTgTgAggCTgTg	21	66.75	57
	reverse	ATgAggTTgAggCTgCTggT	20	66.28	55

oligo	position	sequence	length (bp)	TM (°C)	GC%
SHIP					
	foward	CCATCTACACgCCTCTCACC	20	64.49	60
	reverse	ATTCATCACgCTCCgTCTTC	20	62.62	50
SHP2					
	foward	AgACAgAATTTggATTTggAAgg	23	61.19	39
	reverse	ATTgggAAgCAggTATTgTTgAg	23	63.17	43
SRC					
	foward	CgAgAAAgTgAgACCACgAAAg	22	63.44	50
	reverse	gTgCggAggTgATgTAgAAg	21	64.89	57
VAV1					
	foward	TTCAggAgACCgAgACAACAA	21	63.47	47
	reverse	CATCTCAAACTgCTCCATCCA	21	62.48	47
MYC					
	foward	CTCggATTCTCTgCTCTCCTC	21	63.82	57
	reverse	TCCTCATCTTCTTgTTCCTCCTC	23	63.45	47
RPL38					
	foward	gTTgCTgCTTgCTgTgAgTg	20	64,8	55
	reverse	CAgATTTggCATCCTTTCgTC	21	62,0	47
CD9					
	foward	gTTCTTCggCTTCCTCTTggT	21	64,8	52
	reverse	ggggCTCATCCTTggTTTTC	20	63,5	55
FLI1					
	foward	ATggATggCAgggAACTgTgT	21	66,4	52
	reverse	gTCggTgTgggAggTTgTATT	21	64,5	52
NFE2					
	foward	ATggAACTgACTTggCAggAg	21	64,5	52
	reverse	TAAgTTgTgggTggTggAggT	21	65,6	52
GATA1					
	foward	CCTgCCTCAACTgTgTgTCC	20	65,5	60
	reverse	CCgCTCTgTCTTCAAAgTCTCC	22	65,2	54
FOG1					
	foward	gAgAAgCCCAAAgAgACCTACC	22	64,4	54
	reverse	gACAggCAgATCAggCACAC	20	65,6	60
PF4					
	foward	TgCAgTgCCTgTgTgTgAAg	20	65,7	55
	reverse	TCAgCgTggCTATCAgTTgg	20	64,7	55
TGFB1					
	foward	TCgCCAgAgTggTTATCTTTTg	22	62,9	45
	reverse	CggTAgTgAACCCgTTgATg	20	63,2	55
AURORAB					
	foward	AAAgAgCCTgTCACCCCATC	20	64,4	55
	reverse	ACgCCCAATCTCAAAgTCATC	21	63,1	47

Supplementary table 1: Design of primers used in the study

PMF patient groups	gene identifier	unigene	gene symbol	name
JAK2 WT				
	1843	Hs.171695	DUSP1	dual specificity phosphatase 1
	7132	Hs.279594	TNFRSF1A	tumor necrosis factor receptor superfamily, member 1a
	2353	Hs.25647	FOS	v-fos fbj murine osteosarcoma viral oncogene homolog
	2885	Hs.444356	GRB2	growth factor receptor-bound protein 2
JAK2 V617F				
	1843	Hs.171695	DUSP1	dual specificity phosphatase 1
	1846	Hs.417962	DUSP4	dual specificity phosphatase 4
	5778	Hs.402773	PTPN7	protein tyrosine phosphatase, non-receptor type 7
	22800	Hs.502004	RRAS2	related ras viral (r-ras) oncogene homolog 2
	4893	Hs.37003	HRAS, KRAS, NRAS	v-ha-ras harvey rat sarcoma viral oncogene homolog
	10125	Hs.511010	RASGRP1	ras guanyl releasing protein 1
	6654	Hs.278733	SOS1	son of sevenless homolog 1
	842	Hs.329502	CASP9	caspase 9, apoptosis-related cysteine peptidase
	3553	Hs.126256	IL1B	interleukin 1, beta
	5879	Hs.413812	RAC1	ras-related c3 botulinum toxin substrate 1
	2316	Hs.195464	FLNA	filamin a, alpha

Supplementary table 2: Genes implicated in the MAPK pathway and found differentially expressed in PMF CD34+ cell transcriptome

					HD	HD	HD	PMF	PMF	PMF	ratio PMF/HD
Upstream adaptator	FYN	NM_153047.1	Homo sapiens FYN oncogene related to SRC, FGR, YES (FYI	NM_153047.1	1,09569585	1,139961		0,37975192	1,17692828	1,0139091	0,766542592
	GAB1	AAC50380.1	Growth factor receptor bound protein 2-associated protein 1, in	I_957334	1,13122761	1,3446213	0,95266455	1,00600231	1,35862625	1,02015221	0,987244413
	GAB2	BAA76737.1	Grb2-associated binder 2, an adaptor involved in cell surface re	I_929519	1,03397489	1,11239743	1,05428982	1,05596614	1,12945783	1,09219456	1,024043897
	GADD45G	O95257	Growth arrest- and DNA-damage-inducible protein gamma, a n	I_931413	0,81827635	0,57676649	0,9396584	1,38102007	0,73858416	0,66988462	1,194794779
	SH2D3A	AAD28244.1	Novel SH2 containing protein 1, a member of the NSP family o	I_960779	0,9317084	0,9944272	0,87863803	0,85954773	0,97832918	1,00814497	1,01470645
	SHB	CAA53091.1	SHB (Src homology 2 domain containing) adaptor protein B, bi	I_931311	1,17359972	1,10153925	1,08036375	1,24005234	1,06531978	1,09370446	1,012985791
	SHC3	BAA12322.1	SHC transforming protein c, a neural-specific adaptor protein c	I_1221833	1,50037563	1,00234222	1,08036375	1,14611971	0,93469518	1,37911344	0,965629231
	VAV1	P15498	Vav 1 oncogene, a guanyl-nucleotide exchange factor that is e	I_1109634	0,8413564	0,92817527	0,95986468	1,05730426	0,86439055	0,86022091	1,019242123
	STK4	Q13043	Serine/threonine kinase 4, a protein kinase that functions in ap	I_961482	1,06370211	1,22171521	1,28647101	0,68928689	1,15034997	1,19198287	0,848744263
	RAC1	NM_006908.2	Homo sapiens ras-related C3 botulinum toxin substrate 1 (rho	NM_006908.2	0,98750657	1,22489822	1,38102007	0,93316835	1,18443167	1,01671684	0,872236651
	RAC1	AAD30547.1	Ras-related C3 botulinum toxin substrate 1, a monomeric GTP	I_930828	0,91341889	1,17278934	1,34927356	0,65569216		0,96085972	0,705818853
	PAK1	AAC24716.1	p21 activated kinase 1, a serine-threonine kinase that is activa	I_930796	0,50350058	1,00354993	0,54789835	2,83074021	1,15422368	0,70291042	2,281260811
	SHOC2	Q9UQ13	Suppressor of clear (soc-2) homolog, has 18 leucine-rich repea	I_933145	0,98717695	1,06345282	1,2126861	0,9209584	0,66754321	0,61136419	0,676063673
	SPEC1	AAH12796.2	Small protein effector 1 of cell division cycle 42 (CDC42), mem	I_931476	1,09541833	1,02986431	1,10634351	0,72710979	0,95726019	1,05574739	0,84790667
	PRKCM	Q15139	Mu isoform of protein kinase C (Protein kinase D), a serine-thr	I_958568	1,01921546	1,12496758	1,03881931	1,00070643	1,2556541	1,137025	1,066095828
RAS	HRAS	P01112	Harvey ras, a GTP-binding protein that transmits signals from (	I_929316	1,19418061	1,1014632	1,08915579	0,9155035	1,20990145	1,15592587	0,969431344
	HRASLS	Q9HDD0	HRAS-like suppressor, may inhibit cell growth likely through th	I_928439	0,52191526	0,43042746	0,46989411	0,25228998	3,20626926	0,66512001	2,899432192
	HRASLS2	Q9NWW9	Highly similar to Hras-revertant gene 107, a protein expressed	I_1002159	1,00951067	1,02145207	0,99663466	1,03803027	1,11640048	1,01636338	1,047297079
	HRASLS3	P53816	Hras revertant 107 protein, functions as a class II tumor suppr	I_929219	0,91402906	0,85510612	0,95279616	1,2870636	0,81262469	1,03726566	1,152473579
	KRAS2	NM_033360.1	V-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog, a sm	I_1221964	0,97707975	1,13707733	1,07976687	0,72443599	0,81903034	0,83368117	0,74427179
	NRAS	P01111	Neuroblastoma RAS viral (v-ras) oncogene homolog, a membe	I_937689	0,78753471	0,96805888	1,12813151	0,97400427	0,81155306	0,68574029	0,856980999
	RASA1	P20936	RAS p21 protein activator 1, negatively regulates the RAS patt	I_957441	1,00350833	1,09315062		0,7402867	1,10705709	0,94530076	0,887966583
	RASA2	Q15283	RAS p21 protein activator 2 (GTPase-activating protein 1m), a	I_1109916	1,36961973	1,19371319	1,19787085	1,13101923	1,2811501	1,09209394	0,931686635
	RASA3	NM_007368.1	Homo sapiens RAS p21 protein activator 3 (RASA3), mRNA	NM_007368.1	1,09071171	0,9158619	0,95778936	0,49203953	0,75718153	0,79515171	0,689649946
	RASD1	AAH18041.1	RAS dexamethasone induced 1, a small GTPase and nucleoti	I_957816	1,05706084	0,9990474				1,4900471	1,449385855
	RASGRF1	Q13972	Ras protein-specific guanine nucleotide-releasing factor 1, actr	I_959906	0,9754163	1,17011893	0,97658491	1,22645044	1,05232525	0,9999668	1,050165382
	RASGRF2	AAD55268.1	Ras protein-specific guanine nucleotide-releasing factor 2, actr	I_1151885	1,18309605	1,05084825	0,95012337	0,88942635	1,14823294	1,07924485	0,978906376
	RASGRP1	AAC97349.1	RAS guanyl releasing protein 1 (calcium and DAG-regulated),	I_964784	0,93725985	0,81859672	0,66404879	1,19014537	0,77535397	0,97064406	1,213329844
	RASGRP1	AAC97349.1	RAS guanyl releasing protein 1 (calcium and DAG-regulated),	I_964784	0,93725985	0,81859672	0,66404879	1,19014537	0,77535397	0,97064406	1,213329844
	RASGRP4	CAD58277.1	RAS guanyl releasing protein 4, a guanyl-nucleotide exchange	I_1109188	1,03958499	1,06755435	0,97746229	1,12204432	1,12168264	1,08267975	1,078391022
	RASSF1	NM_170715.1	Ras association domain family 1, a tumor suppressor protein t	I_950067	0,89765614	0,83245355	0,80736518	1,58416355	1,00309944	1,02066207	1,421856468
	RASSF6	NM_177532.1	Homo sapiens Ras association (RalGDS/AF-6) domain family	NM_177532.1	1,16404557	1,18508637	1,13360047	1,10009456	1,11920595	1,32831168	1,018628986
	RRAS	P10301	Related RAS viral oncogene homolog, inhibits apoptosis throu	I_961994	0,94050258	0,97755903	1,15995193	1,09420824	0,96691877	0,84820336	0,945197391
	RRAS2	P17082	Small GTP-binding RAS-related GTPase, overexpression can t	I_930781	0,83180207	0,54789835	0,62748015	0,34641767	2,44118118	1,54170048	2,156905757
RAF	ARAF1	P10398	A-Raf proto-oncogene (v-raf murine sarcoma 3611 viral oncoge	I_962111	1,09355342	0,78144783	0,93280315	0,92557156	0,92789751	0,73960531	0,923523867
	ARAF1	P10398	A-Raf proto-oncogene (v-raf murine sarcoma 3611 viral oncoge	I_962111	1,09355342	0,78144783	0,93280315	0,92557156	0,92789751	0,73960531	0,923523867
	BRAF	NM_004333.1	B Raf, a member of the Raf family of serine/threonine kinases,	I_1110149	1,0074836	0,98762709	1,02841878	1,05776691	1,16415286	1,05915618	1,085180742
	BRAF	NM_004333.1	B Raf, a member of the Raf family of serine/threonine kinases,	I_1110149	1,0074836	0,98762709	1,02841878	1,05776691	1,16415286	1,05915618	1,085180742
	CBLC	Q9ULV8	Cas-Br-M (murine) ectropic retroviral transforming sequence c,	I_1110013	1,0766139	1,20146191	0,99663699	1,08250523	1,05633092	1,10512137	0,990608251
	CDC42	NM_044472.1	Cell division cycle 42 (GTP binding protein 25kD), Rho GTPase	I_942693	0,91432375			1,00359845	1,1105481		1,156129474
	CDKL1	Q00532	Cyclin-dependent kinase like 1 (CDC2-related kinase 1), meml	I_958677	1,33540308	1,31513047	1,35177028	1,32707345	1,23906696	1,27649736	0,960106462
	DAB2	P98082	Disabled homolog 2, binds MADH2, MADH3 and GRB2, uncou	I_958462	0,79213643	1,11457682	1,13574278	0,93771315	1,12145019	0,91784638	0,978488985
	RAF1	P04049	V-raf-1 murine leukemia viral oncogene homolog 1, protein kina	I_928612	0,96105891	0,8547911	0,92057675	1,24746966	0,97809941	0,96874619	1,167330801
MAPK	I_943702	AAF03789.1	Protein with high similarity to mouse Map4k6, which activates	I_943702	0,99401057	1,06910408	1,0685873	0,86758095	1,16557443	1,00915527	0,971456003
	I_962966	Q99683	Protein with high similarity to human MAP3K5, which is MAP	I_962966	1,09629905	1,11209869	1,13161314	0,7377497	1,13209593	1,07535863	0,881794809
	KIAA1277	BAA86591.1	Protein with high similarity to suppression of tumorigenicity 5 (	I_1152066	0,846389	0,83201277	1,09531748	0,8635152	1,11694038	0,923379	1,046910058
	KIAA1361	BAA92599.1	Protein with very strong similarity to thousand and one amino a	I_960303	1,0751276	1,01659739	0,96694106	1,26269948	1,60768151	0,88131267	1,226578386
	MADD	NM_003682.2	MAP-kinase activating death domain, guanyl nucleotide excha	I_929406	1,01723671	0,99855798	0,99301088	0,90304625	1,05065465	1,05535841	1,000084331
	MAP2K1	Q02750	Mitogen-activated protein kinase kinase 1, activates the MAP I	I_929473	0,9396584	0,94408262	0,83383477	1,34555054	0,93754041	0,8752256	1,162181591
	MAP2K1IP1	Q9UHA4	Mitogen-activated protein kinase kinase 1 (MEK1) interacting p	I_962208	1,00709629	1,09086251	1,23333204	1,23213983	0,88715601	0,70680565	0,848350269
	MAP2K3	NM_145110.1	Mitogen activated protein kinase kinase 3, phosphorylates MA	I_957882	0,90521139	0,93766993	0,93331879	0,89106625	1,0870012	1,00767624	1,07547856
	MAP2K5	AAH08838.1	MAP kinase kinase 5, a member of the MAP kinase family tha	I_1152573	1,76847935	1,90458345	1,54988694	1,3384434	3,24414301	1,1729444	1,101965573
	MAP2K6	P52564	Mitogen activated protein kinase kinase 6, MAP kinase kinase	I_959681	0,97889924	1,15475535	0,85622913	0,81748539	0,84054315	0,94804728	0,871631162
	MAP2K7	NM_005043.2	Mitogen activated protein kinase kinase 7, a MAPK kinase tha	I_961642	0,6397348	0,88658422	0,56402713	0,72794741	0,7504124	0,78777099	1,084093559
	MAP3K3	Q99759	Mitogen activated protein kinase kinase kinase 3, activates the	I_958401	0,85617	0,83546835	0,76313257	1,64361465	1,27262366	0,99500442	1,593322899
	MAP3K4	Q9Y6R4	MAP kinase kinase kinase 4, enzyme that phosphorylates MK	I_957010	1,13906896	1,09491396	1,1191802	0,95792168	1,12831402	1,19280648	0,977895218
	MAP3K7IP1	NM_006116.1	TAK binding protein 1, exhibits ubiquitin-dependent activation c	I_961952	1,06394696	0,96335427	1,01576447	1,07539988	1,05652559	1,06379998	1,043309464
	MAP3K7IP2	BAA34453.2	TAK1-binding protein 2, an adaptor protein that functions in the	I_957418	0,99435395	0,96375058	0,93465215	0,7504124	0,86630338		0,83256995
	MAPK1	P28482	Mitogen-activated protein kinase 1, a serine-threonine kinase e	I_961154	1,04116607	1,03321338	1,06289411	0,53345776	0,89340836	0,84209287	0,723226378
	MAPK11	NM_002751.4	Homo sapiens mitogen-activated protein kinase 11 (MAPK11),	NM_002751.4	0,81481683	0,83320141	0,90918028	0,8912099	0,91994107	1,2216872	1,18600028
	MAPK11	Q15759	Mitogen-activated protein kinase 11, a MAP kinase that is acti	I_961883	1,04650187	1,00742567		1,10202134	0,93838269	1,02924788	0,996351897
	MAPK13	O15264	Mitogen activated protein kinase 13, a p38 MAP kinase that is	I_957351	1,36458313	0,89047146	1,21394777	1,68151212	2,18625093	1,78648758	1,629935649
	MAPK4	P31152	Mitogen activated protein kinase 4, a member of the MAP kina	I_960583	1,51356125	3,83442283	0,76789176	1,93508482	2,59896231	1,23816919	0,943808617
	MAPK7	Q13164	Mitogen activated protein kinase 7 (big mitogen activated prote	I_958691	1,07031107	0,94643325	0,93918252	1,16893399	0,94006956	1,00113463	1,052170216
	MAPK8	NM_002750.2	Homo sapiens mitogen-activated protein kinase 8 (MAPK8), tr	NM_002750.2	0,91879791	0,89699495	0,99292624	0,84732497	0,87014174	0,83466077	0,908644614
	MAPK8IP2	NM_016431.2	Homo sapiens mitogen-activated protein kinase 8 interacting p	NM_016431.2	0,91341889	0,81387931	0,90475291	0,98901528	1,03822148	0,99002922	1,146355389
	MAPK8IP2	Q13387	Mitogen-activated protein kinase 8 interacting protein 2, a scaf	I_962004	1,1134994	1,1228714	1,06223357	1,16367042	1,16436732	1,12181175	1,045851245
	MAPK8IP3	NM_015133.1	Protein with strong similarity to mitogen-activated protein kinas	I_958669	0,93600893	0,77553248	0,67483872	1,03934562	1,05431402	1,28233063	1,414690904
	MAPK8IP3	NM_033392.1	Homo sapiens mitogen-activated protein kinase 8 interacting p	NM_033392.1	0,88975412	0,9055866	0,85544086	0,78758967	0,86334429	0,94632429	0,98735342
	MAPKAPK2	NM_004759.2	Homo sapiens mitogen-activated protein kinase-activated prote	NM_004759.2	1,32129562	1,23219657	1,22939086	1,22165895	1,29897261	1,70962155	1,118261666
	MAPKAPK3	AAH07591.1	Mitogen-activated protein kinase-activated protein kinase 3, pu	I_950167	1,15247095	1,03939354	1,08530045	0,92960835	1,03731346	0,92791885	0,883336882
	MAPKAPK5	AAH41049.1	MAP kinase activated protein kinase 5 (p38-regulated/activate	I_935383	0,99732566	0,95080179	1,0071032	0,86385328	0,93142951	0,85551965	0,896986647
	MBIP	BAA94083.1	MAP kinase upstream kinase-binding inhibitory protein, a prote	I_958794	1,04178953	1,43979275	1,38197434	1,16166258	1,29092205	0,92804706	0,875005083
	MIG-6	Q9UJM3	Mitogen-inducible gene 6, putative tumor suppressor expresse	I_1152475	0,77911216	1,05696356	1,03891492	1,24893546	1,46521044	1,01159346	1,295913561
	MKNK1	NM_003684.2	MAP kinase-interacting serine-threonine kinase 1, phosphoryla	I_928775	1,04592371	1,04616451	1,06062043	1,44577265		0,86206496	1,098026108
	MKNK2	AAG26337.1	MAP kinase interacting serine threonine kinase 2, a protein ki	I_966310	1,02039194	1,0033443	0,93570727	1,09662638	1,05788875	1,05696356	1,085838832
	MKP-7	Q9BY84	MAPK phosphatase-7, a cytoplasmic protein phosphatase tha	I_944483	1,07332194	1,25216055	1,12096551	1,17462707	1,20035577	1,01604509	0,983913947
	NM_002747.2	NM_002747.2	Homo sapiens mitogen-activated protein kinase 4 (MAPK4), m	NM_002747.2	1,1301862	1,40637136	1,1289897	1,25305462	1,54846001	1,25695574	1,107193576
Phosphatases	DUSP10	Q9Y6W6	Dual specificity phosphatase 10, a MAP kinase phosphatase t	I_965121	0,9642064	0,91276717	0,93198729	1,3384434	1,03488004	1,03354645	1,212857731
	DUSP15	NM_080611.2	Protein with high similarity to mitogen-activated protein (MAP)	I_960977	1,1614486	1,20162785	1,05096924	1,08762705	0,89431393	1,13754869	0,913722297
	DUSP18	Q8NEJ0	Protein with high similarity to dual specificity phosphatase 14	I_961410	0,94743627	1,31886399	2,77843356	0,96325219	0,97341943	0,96061641	0,574319305
	DUSP21	NM_022076.1	Protein with high similarity to dual specificity phosphatase 14	I_962364	2,55976439	0,42501083	0,99055594	0,62646961	0,63503844	1,60953343	0,722214418
	DUSP3	P51452	Dual specificity phosphatase 3 (vaccinia H1 related phosphata	I_1100517	0,69119406	0,93728143	0,96990681	1,31643677	0,89456105	0,89248312	1,194389656
	DUSP4	Q13115	Dual specificity protein phosphatase 4, inactivates MAP kinas	I_929318	0,99267024	1,00972247	0,99549478	1,39219475	1,07193887	1,12116623	1,195942096
	DUSP5	NM_004419.2	Dual specificity phosphatase 5, member of a family of nuclear	I_933139	0,6600852	1,33444297	1,29658198	0,37471408	0,8965407		0,579403932
	DUSP9	Q99956	Dual specificity phosphatase 9, inactivates mitogen-activated p	I_962398	0,90825963	0,95104265	0,98134792	0,86419153	0,99331045	0,84009808	0,949641764
	DKFZp761G0	CAD38946.1	Member of the protein phosphatase 2C family, which are Mg2+	I_957533	1,13631833	1,30677259	1,2321682	0,50827646	0,92008936	0,9861092	0,656953687
	PTPN7	NM_080588.1	Protein tyrosine phosphatase non-receptor type 7 (hematopoie	I_964350	0,99767482	1,02440107	1,11745489	0,53926224	1,09179223	0,88334429	0,800883613
downstream TFs	EHF	AAD30991.1	ETS homologous factor, a transcription factor that is a membe	I_935483	0,88589042	1,26321671	0,76630205	0,89555031	1,12266457	0,89016396	0,990791625
	ELK1	P19419	Protein of the Ets oncoprotein family; rearrangements of the cc	I_962100	1,01129067	0,99934119	1,02144969	0,96562633	1,03067088	1,02035666	0,994912511
	ELK3	P41970	SRF accessory protein 2 (SAP2), a member of the Ets family	I_943954	1,10202134	1,06382453	1,0556258	1,03200066	1,07567239	1,04157364	0,97758013
	ELK4	P28324	SRF accessory protein 1, a member of the ETS family of trans	I_936147	1,32281768	1,83189249	1,31795323	0,99599922	0,79493201	0,88242948	0,597711132
	FLJ23074	NM_025052.1	Protein containing a protein kinase domain, has high similarity	I_928482	1,03669262	1,26182759	1,08632553	1,07154393	1,48696244	1,15496814	1,097088259
	FLJ23074	NM_025052.1	Protein containing a protein kinase domain, has high similarity	I_928482	1,03669262	1,26182759	1,08632553	1,07154393	1,48696244	1,15496814	1,097088259
	FOS	P01100	V-fos homolog, transcription factor that forms a heterodimer wi	I_959033	0,71597856	0,56182361	0,45488331	3,19080305	0,75631028	1,07554853	2,898772993
	FOSB	P53539	DNA-binding member of the Fos family; forms AP-1 transcripti	I_962545	0,97855222	0,91230488	0,97613525	0,65042913	1,16818058	0,99333334	0,980798937
	FOSL1	P15407	FOS-like antigen 1, a member of the Fos family of proteins tha	I_1110036	1,09860098	1,04988086	1,17945123	0,99864656	1,12953579	1,01333261	0,943983815
	FOSL2	P15408	FOS-like antigen 2, a member of the Fos protein family that he	I_943963	0,95411342	0,8751651	0,83581471	1,07226446	0,91066784	0,97633755	1,11038886
	JUN	P05412	V-jun homolog, proto-oncoprotein and transcription factor that	I_931585	0,32486281	0,21612251	0,29709816	4,68273926	0,2763758	0,43752211	6,439259725
	JUNB	P17275	Jun B proto-oncogene, member of the Jun family of DNA bindir	I_962124	0,96469492				1,2361182		1,281356598
	JUND	P17535	Jun D proto-oncogene, a member of the Jun family of DNA binc	I_966589	1,30918193	1,27997065	1,08322835	0,91668475	0,92013168	1,0248965	0,77925275

Supplementary table 3 : MAPK pathway genes in PMF and HD MK transcritpome

gene	samples	H0	H1	H2	H4	H6	H18	conclusion
ATF2								
	HD	2,27 +/- 1,77	1,14 +/- 0,35	1,15 +/- 0,17	1,44 +/- 0,12	1,51 +/- 0,15	1,81 +/- 0,43	down-regulation
	PMF	0,67 +/- 0,13	0,72 +/- 0,26	0,71 +/- 0,17	0,91 +/- 0,21	1,08 +/- 0,16	0,99 +/- 0,23	up-regulated progressively
AP-1								
	HD	12,27 +/- 17,11	9,74 +/- 11,94	3,93 +/- 4,18	2,48 +/- 2,87	2,80 +/- 3,22	3,61 +/- 2,18	down-regulation
	PMF	0,98 +/- 0,38	1,94 +/- 1,65	0,44 +/- 0,46	0,51 +/- 0,59	0,51 +/- 0,51	0,54 +/- 0,24	up-regulated at 1 hour
MSK2								
	HD	0,58 +/- 0,61	1,33 +1,33	0,95 +/- 0,61	0,76 +/- 0,12	1,38 +/- 0,22	1,13 +/- 0,55	lightly up regulated
	PMF	1,18 +/- 0,82	1,62 +/- 0,96	1,33 +/- 0,78	1,83 +/- 2,26	1,18 +/- 0,62	1,13 +/- 0,64	lightly up regulated
IL-8								
	HD	24,61+/- 31,28	23,32 +/- 31,75	8,98 +/- 11,31	7,56 +/- 10,07	17,76 +/- 24,62	8,59 +/- 10,42	down-regulation
	PMF	0,68 +/- 0,64	3,49 +/- 4,05	1,16 +/- 12	1,38 +/- 2,1	0,35 +/- 0,31	0,19 +/- 0,12	up-regulated at 1 hour
MEFC2								
	HD	3,05 +/- 2,45	1,46 +/- 0,92	1,60 +/- 0,54	1,80 +/- 0,59	2,41 +/- 0,84	1,90 +/- 0,40	down-regulation
	PMF	0,59 +/- 0,31	0,73 +/- 0,43	0,79 +/- 0,40	0,71 +/- 0,18	0,86 +/- 0,43	0,87 +/- 0,65	stable
MK3								
	HD	1,33 +/- 0,17	1,31 +/- 0,11	1,04 +/- 0,09	0,90 +/- 0,07	1,17 +/- 0,18	1,29 +/- 0,52	stable
	PMF	0,91 +/- 0,29	1,19 +/- 0,30	1,21 +/- 0,33	0,93 +/- 0,50	0,95 +/- 0,17	0,61 +/- 0,13	stable
MNK1								
	HD	1,04 +/- 0,20	1,78 +/- 0,07	1,46 +/- 1,24	0,95 +/- 0,75	0,88 +/- 0,25	1,44 +/- 0,45	stable
	PMF	1,17 +/- 0,56	1,25 +/- 0,32	1,01 +/- 0,32	0,78 +/- 0,27	0,79 +/- 0,06	0,83 +/- 0,14	stable
MNK2								
	HD	0,98 +/- 0,37	0,95 +/- 0,18	0,53 +/- 0,05	0,48 +/- 0,12	0,52 +/- 0,26	0,83 +/- 0,46	stable
	PMF	1,40 +/- 0,24	1,80 +/- 1,12	1,45 +/- 0,24	1,08 +/- 0,37	1,22 +/- 0,14	1,31 +/- 0,12	stable
MSK1								
	HD	1,34 +/- 0,10	1,89 +/- 0,29	1,59 +/- 0,01	1,28 +/- 0,42	1,74 +/- 0,52	1,28 +/- 1,13	stable
	PMF	0,64 +/- 0,12	0,70 +/- 0,13	0,82 +/- 0,38	1,05 +/- 0,58	0,80 +/- 0,32	0,98 +/- 0,13	stable
NFATC4								
	HD	2,80 +/- 1,37	1,50 +/- 0,81	1,26 +/- 0,73	1,19 +/- 0,73	1,86 +/- 1,30	1,68 +/- 1,17	down-regulation
	PMF	1,40 +/- 0,61	1,08 +/- 0,51	2,60 +/- 1,36	2,14 +/- 1,34	2,21 +/- 1,24	1,74 +/- 2,12	up-regulated at 2 hour
P53								
	HD	1,12 +/- 0,11	0,71 +/- 0,28	0,72 +/- 0,11	0,57 +/- 0,32	0,57 +/- 0,26	0,68 +/- 0,41	down-regulation
	PMF	0,96 +/- 0,18	1,96 +/- 0,86	1,67 +/- 0,59	1,34 +/- 0,18	1,30 +/- 0,26	1,00 +/- 0,06	up-regulated at 1 hour
PARK								
	HD	1,31 +/- 0,40	1,01 +/- 0,87	1,30 +/- 0,05	1,28 +/- 0,28	1,55 +/- 0,29	1,18 +/- 0,38	stable
	PMF	0,82 +/- 0,28	0,97 +/- 0,20	1,06 +/- 0,01	1,11 +/- 0,52	0,73 +/- 0,24	0,69 +/- 0,28	stable
MK2								
	HD	NA	NA	NA	NA	NA	NA	not expressed
	PMF	NA	NA	NA	NA	NA	NA	not expressed
ETS-1								
	HD	NA	NA	NA	NA	NA	NA	not expressed
	PMF	NA	NA	NA	NA	NA	NA	not expressed
CHOP								
	HD	NA	NA	NA	NA	NA	NA	not expressed
	PMF	NA	NA	NA	NA	NA	NA	not expressed

Supplementary table 4: Modulation of transcripts for MAPK downstream regulators in MK-derived from CD34+ cells in response to FL stimulation

	PMF Patients				Healthy donors		
	PMF1	PMF2	PMF3	PMF4	BM	BM	PB
RAC1	4.027069368	8.492133838	3.13639066165322	3.77817250273135	3.74786623988852	3.02458899179157	3.93233778662723
CDC42	3.30420295517666	8.5794908296441	6.45368654410474	3.6246254771943	4.16331698878299	4.18579236072083	3.68215214885103
BCL2	0.888174793943578	6.45650333401331	NA	3.15319943969532	2.26284118009991	NA	2.62790636726842
GAB1	3.0305053702145	9.387041878604 09	19.4989382179185	3.6500356811567	3.40611984792922	3.96818475352986	4.01929580603241
GRB2	5.5037798847019	23.6528894380285	6.86852969704324	2.61577139924021	4.53766616961476	2.32276058763806	3.69488099576216
SHC1	2.64588897951582	14.974646177764	6.13712317321595	4.49383697704643	3.29303499045515	2.07270830284608	4.1700775436863
BCLXL	1.96093961867809	5.44409379682158	3.33662568302991	1.75150450192655	2.93700058909115	1.48945007537135	2.71850743652894
MAP3K1	1.19756418450003	24.6947036029108	12.049083664556	3.64978167781552	6.46830744970123	5.1100150525007	6.26164367642963
MYC	0.862216671978366	53.9373527668646	0.982837685193087	1.06457114954803	0.851900054432574	2.30665122345216	1.35767103040781
MCL1	1.96600686794509	3.69799145682522	6.73285957851515	3.82076004748081	9.43986068357189	54.925027406225	18.8882357346777
p85	1.77832361196893	23.9555700138644	3.46977699254579	5.10864735992192	5.06944349112013	5.17188344509695	5.89773462104031
SRC	3.87659992956336	8.71067800149246	4.97353737595091	3.68565854011991	4.09753288559306	4.15614564594006	4.07970934924142
PAK1	2.20531788909879	5.42620234428608	3.20902097757171	2.57313122270652	2.54123222053718	3.14126680382895	4.39260910518999
VAV1	8.61222077391724	47.2824582769769	7.33852129088751	3.62856857589125	3.14181575680558	4.61155093188347	4.33698845039068

Hemoglobin (g/dl)	5.9	10.6	9.8	8.9
Platelets ($X10^3/mm^3$)	74	674	146	61
Leukocytes ($10^3/L$)	3970	20670	2830	8900
Jak2 mutation	V617F	wt	V617F	V617F
Dupriez score	2	0	2	1
Summary	PMF jak2V617F Dupriez(2) Hypoleukocytosis	PMF jak2WT Dupriez(0) Hyperleukocytosis	PMF jak2V617F Dupriez(2) Hypoleukocytosis	PMF jak2V617F Dupriez(1) Normal Leukocytosis

Supplementary table 5: QPCR cumulative ratio of transcripts modulated during the 18 hour FL stimulation kinetic and clinico-biological data of patients and healthy donors

inhibitor	target	concentration	patients tested	condition	ploidy	B/S ratio
PD98059	ERK 1/2	25 µM	13			
				control	8,35 +/- 5,82	3,64 +/- 3,16
				traited	11,56 +/- 7,02	5,03 +/- 3,99
					p < 0,0001	p = 0,02
2-acylaminothiophene-3-carboxamide-3,4-Di-Ome	ATP-binding pocket of FLT3	42 nM	5			
				control	7,82 +/- 6,71	17,4 +/- 7,61
				traited	10,26 +/- 6,02	27,00 +/- 14,65
					p = 0,02	NS
4-(4-Aminophenyl)-1H-indazol-3-ylamine	ATP-binding pocket of FLT3	50 nM	6			
				control	10,73 +/- 5,32	10,49 +/- 6,26
				traited	13,90 +/- 6,62	14,47 +/- 8,39
					p = 0,002	p = 0,006
Anti-human FLT3 Mab (clone 66907)	FLT3 extracellular domain	10 µg/ml	9			
				control	10,83 +/- 4,39	4,87 +/- 4,59
				traited	14,12 +/- 5,98	7,62 +/- 7,55
					p = 0,004	p = 0,03
PD169316	p38 alpha-beta-gamma-delta	90 nM	7			
				control	11,29 +/- 4,42	10,66 +/- 5,92
				traited	13,83 +/- 8,41	15,16 +/- 12,52
					NS	NS
SB203580	p38 alpha-beta	1 µM	7			
				control	11,29 +/- 4,42	10,66 +/- 5,92
				traited	15,27 +/- 8,70	13,79 +/- 7,19
					p = 0,05	p = 0,01
SB202190	p38 alpha-beta	40 nM	6			
				control	11,39 +/- 4,84	10,67 +/- 6,49
				traited	14,11 +/- 7,05	10,70 +/- 6,73
					p = 0,05	NS
SP600125	JNK 1,2,3	100 nM	6			
				control	11,20 +/- 4,84	10,30 +/- 6,41
				traited	14,34 +/- 8,82	11,31 +/- 7,29
					NS	NS

Supplementary table 6: In vitro effect of agents targeting FLT3/MAPK axis on PMF MK ploidy

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